.

THE JOURNAL

OF

BIOLOGICAL CHEMISTRY

FOUNDED BY CHRISTIAN A. HERTER AND SUSTAINED IN PART BY THE CHRISTIAN A. HERTER
MEMORIAL FUND

OFFICIAL ORGAN OF THE AMERICAN SOCIETY OF BIOLOGICAL CHEMISTS

EDITED BY

STANLEY R. BENEDICT, New York, N. Y. LAFAYETTE B. MENDEL, New Haven, Conn.
HENRY D. DAKIN, Scarborough, N. Y.   DONALD D. VAN SLYKE, New York, N. Y.

WITH THE COOPERATION OF THE EDITORIAL COMMITTEE

OTTO FOLIN, Boston, Mass.
L. J. HENDERSON, Cambridge, Mass.
ANDREW HUNTER, Toronto, Canada

WALTER JONES, New York, N. Y.
GRAHAM LUSK, New York, N. Y.
THOMAS B. OSBORNE, New Haven, Conn

WALTER W. PALMER, New York, N. Y.
A. N. RICHARDS, Philadelphia, Pa.
L. L. VAN SLYKE, Geneva, N. Y.

VOLUME LIII
BALTIMORE
1922

CONTENTS OF VOLUME LIII.

No. 1. July, 1922.

Contents

No. 2. August, 1922.

Contents

V

OBSERVATIONS ON THE PRESENCE OF THE ANTI-NEURITIC SUBSTANCE, WATER-SOLUBLE B, IN CHLOROPHYLL-FREE PLANTS.

By C. R. ORTON,

(*Contribution No. 37 from the Department of Botany, The Pennsylvania State College, State College.*)

and E. V. McCOLLUM and NINA SIMMONDS.

(*From the Department of Chemical Hygiene, School of Hygiene and Public Health, Johns Hopkins University, Baltimore.*)

The numerous studies of the last few years have shown that the dietary factor, water-soluble B, is almost, if not quite, universally distributed among natural food substances of vegetable origin, although there are wide variations in the amounts of it which are furnished by different tissues. Thus, the germ of wheat is extraordinarily rich in this vitamin, whereas the onion bulb proved inadequate for the protection of the Philippine Scouts against beri-beri in 1910–15 (1), and McCarrison (2) found that pigeons developed typical beri-beri on a diet containing liberal amounts of onion, indicating that the onion bulb is extremely deficient in water-soluble B.

Osborne and Mendel (3) have reported experiments which indicate that rats which have been brought to a condition where they were declining as the result of lack of water-soluble B, responded well when they were fed onion in a liberal amount. It appears from their observations that the onion is not entirely free from this vitamin. In our own laboratory we have tested onion with similar results (4).

There is no difficulty about securing foods which are entirely lacking in fat-soluble A and water-soluble C, the antiophthalmic and antiscorbutic substances respectively, but it is very difficult to secure sources of the factor C, which do not contain the factor B. For the purpose of enabling investigators to prepare easily

1

rations which are suitable for inducing uncomplicated beri-beri, it is very desirable that we have some readily obtainable articles of diet which possess no water-soluble B, but an abundance of water-soluble C. We have, accordingly, given some attention to examining unusual or highly specialized plant tissues, in the hope of placing at the disposal of students of nutrition, foodstuffs with these unique properties.

We have sought to test the question whether in plant tissues the vitamin, water-soluble B, is associated directly with the chloroplasts. Etiolated leaves appear from our studies to be as effective sources of water-soluble B as are leaves in which chlorophyll has been caused to develop as the result of illumination. The possibility still remains, however, that the vitamin may be associated with the chloroplast which is present in the leaf, even though chlorophyll exists only as a precursor which quickly transforms in the presence of light. The roots of the onion contain no chloroplasts, and it was thought worth while to test these for their antineuritic properties.

When the bases of onion bulbs are immersed in water under suitable conditions of temperature they send out numerous roots. It is easy, although time-consuming, to secure considerable amounts of this plant tissue, which contain none of the anatomic structures concerned with photosynthesis.

Our tests have been made by the following procedure: Young rats weighing about 50 gm. were restricted to a diet consisting of

	per cent
Casein	18.0
Salt mixture (185)	3.7
Agar-agar	2.0
Dextrin	71.3
Butter fat	5.0

On this diet they may increase slightly in weight during the first 2 or 3 weeks, but thereafter they steadily decline in weight and lose their muscular strength. Eventually, unless the dietary factor water-soluble B, which is the sole deficiency in this food, in as far as the needs of the rat are concerned, is added, death supervenes, with or without the development of acute symptoms of beri-beri. It is best to administer the substance whose vitamin content it is desired to determine before the animals have

deteriorated too far physically. In practice we have allowed the
animals to remain on the deficient diet until their appearance
and loss of weight showed clearly that they were repeating the
usual behavior of animals on diets free from water-soluble B.
We then modified the diet by replacing a part of the dextrin by
the food substance which we desired to study.

Test of Onion Root for Water-Soluble B.

Two young rats were prepared as above described for testing
the value of onion roots for water-soluble B. On the 29th day
the diet was modified by the introduction of 4.4 per cent of dry
onion roots. One animal died almost as soon as this change was
made. The other responded in a manner which is illustrated by
the curve in the accompanying chart (Lot 2973 D). It is evident
that onion roots contain a small amount of water-soluble B, but
not more than a similar amount of a cereal grain. It would re-
quire about 15 to 20 per cent of whole wheat to cause the recovery
and resumption of growth in a rat in the condition of the one
described. About 3 per cent of wheat germ would suffice to bring
about this result.

A Test of a Mushroom (Agaricus campestris) for Water-Soluble B.

Two young rats were prepared in the usual way by restricting
them to the diet which was satisfactory in all respects except
that it lacked water-soluble B. At the end of the 4th week both
were definitely declining. On the 29th day, 9 per cent of dried
Agaricus campestris was introduced into the food formula in place
of an equivalent amount of dextrin. No other change was made
in the feed or management of the animals. Their response with
increased physical strength and improved appearance was immed-
iate. They began at once to grow, and continued to do so to
the end of the test, which lasted until one had increased in weight
from 85 to 148 gm. The other increased from a body weight of
70 to 115 gm. The mushroom feeding period extended over 5
weeks. This indicates that the mushroom is an excellent source
of water-soluble B. Coward and Drummond (5) have recently
reported that Agaricus campestris is almost devoid of fat-soluble A.

Ration of all groups in Period 1.

Casein	18.0
Salts (185)	3.7
Agar-agar	2.0
Dextrin	71.3
Butter fat	5.0

Period 2.

Lot 3263 C.
9 per cent of dry mushrooms replaced part of dextrin

Lot 3263 C

Period 2.

Lot 3264 A.
3 per cent of dry Indian-pipe replaced part of dextrin

Lot 3264 A

Period 2.

Lot 3304 A.
15 per cent of dodder replaced part of dextrin

Lot 3304 A

Period 2.

Lot 2973 D.
4.4 per cent of onion sprouts replaced part of dextrin

Lot 2973 D

GRAMS

160

120

80

40

< 4 >
Weeks

CHART 1.

4

Test of Indian-Pipe (Monotropa uniflora) for Water-Soluble B.

Our supply of material was limited to 20 gm. of the dry substance of this plant. We, therefore, limited our test to a single animal. This was restricted as above described to the experimental diet to bring it into a condition where its body was depleted of water-soluble B. On the 29th day, when it was declining in vigor in the usual manner, the diet was modified so as to include 8 per cent of *Monotropa uniflora* in place of an equivalent amount of dextrin. There was no response with growth, but the condition of the animal improved, and it remained active over a period of 7 weeks. At this point the experimental ration was exhausted and the test was discontinued. The experiment is inconclusive, but indicates that *Monotropa uniflora* probably contains a moderate amount of water-soluble B for the rat's life was prolonged beyond the average of those which we have seen restricted to this diet without a source of water-soluble B.

Test of a Non-Chlorophyll-Producing Parasitic Plant (Cuscuta gronovii) for Water-Soluble B.

Gronovius' dodder (*Cuscuta gronovii*), is a parasitic plant which is devoid of chlorophyll, and can be secured in large amounts with little difficulty. It was, therefore, thought worth while to test it for its content of water-soluble B. To this end we restricted six young rats to the deficient diet until they were in a state of decline owing to specific starvation for water-soluble B. About the 35th day 15 per cent of dry dodder was introduced into the diet in place of an equivalent amount of dextrin. The animals all died within a week after this change was made, apparently because of toxicity of the dodder.

CONCLUSIONS.

We have tested the onion root, a structure which contains no chloroplasts, for the presence of water-soluble B, and have found it to contain a certain amount of this dietary essential. This warrants, we believe, the conclusion that the substance, water-soluble B, is not concerned with the structure of the chloroplast.

The mushroom, *Agaricus campestris*, proved to be a good source of water-soluble B.

Indian-pipe, *Monotropa uniflora*, a non-chlorophyll-bearing plant, gave results which were inconclusive when tested by our method for the presence of water-soluble B.

Dodder, *Cuscuta gronovii*, proved toxic, and caused the death of the experimental animals. It cannot be determined from our experiments whether this parasitic plant does or does not contain water-soluble B.

BIBLIOGRAPHY.

1. Chamberlain, W. P., Prevention of beriberi among Philippine scouts by means of modifications in the diet, *J. Am. Med. Assn.*, 1915, lxiv, 1215.
2. McCarrison, R., The effects of deprivation of "B" accessory food factors, *Indian J. Med. Research*, 1918–19, vi, 550.
3. Osborne, T. B., and Mendel, L. B., Nutritive factors in plant tissues. II. The distribution of water-soluble vitamine, *J. Biol. Chem.*, 1919, xxxix, 29.
4. McCollum, E. V., and Simmonds, N., Unpublished data.
5. Coward, K. H., and Drummond, J. C., The formation of vitamin A in living plant tissues, *Biochem. J.*, 1921, xv, 530.

GLACIAL ACETIC ACID AS A SOLVENT FOR THE ANTI-NEURITIC SUBSTANCE, WATER-SOLUBLE B.[*]

By VICTOR E. LEVINE,

(From the Creighton Medical School, Creighton University, Omaha.)

AND E. V. McCOLLUM AND NINA SIMMONDS.

(From the Department of Chemical Hygiene, School of Hygiene and Public Health, Johns Hopkins University, Baltimore.)

Up to the present time water has proved the only effective solvent for the antineuritic substance, water-soluble B. It is usually stated that this substance is also extracted from natural foods by hot alcohol, but this is, strictly speaking, not true, for absolute alcohol does not extract a sufficient amount of the substance from such vitamin-rich substances as wheat germ to give satisfactory results in growth experiments on young rats. Alcohol containing water is a solvent for the vitamin, and indeed, the higher the content of water, the better the solvent action of the alcohol.

Benzene, alcohol, ether, ethyl acetate, and acetone have been tested thoroughly as solvents for water-soluble B, employing the rats as the test organism (1). Our later studies have shown us that while it is possible to extract from the alcoholic extract of natural foods by means of hot benzene a sufficient amount of water-soluble B to give a positive test for the vitamin, this solvent is too poor to have any practical value as an aid to the isolation of the substance.

McCollum and Simmonds (1) have discussed the relative merits of the rat and the pigeon as subjects for testing the potency of any preparation supposed to contain this substance. It is their view that the only satisfactory method of conducting this test

---

[*] Since this paper was sent to the publishers we have noted in the Vitamine Manual by W. H. Eddy that he has used glacial acetic acid as a solvent for water-soluble B.

is to restrict a young rat for a period to a diet which is satisfactory in all respects except for the lack of water-soluble B, and to pass judgment as to the presence or absence of the vitamin in question in a given preparation by the response or failure of response with growth after the animal has declined to a point near collapse. If a rat, in an enfeebled condition, under such conditions returns on the administration of a preparation of water-soluble B to a state of vigor, resumes growth at normal rate, and exhibits a normal appearance, there can be no doubt that the vitamin in question has been supplied. On the other hand, there is much evidence that the traditional test for this vitamin, using the pigeon as a subject, is unreliable, and may lead to serious errors in judgment concerning the properties of various preparations which may be made for the study of this most interesting substance. Dutcher (2) has pointed out that physiological stimulants of several kinds, which McCollum and Koch (3) have shown to possess no power to replace the substance water-soluble B, may induce temporary improvement in a pigeon suffering from acute polyneuritis. McCollum and Simmonds (4) have convinced themselves that several of the substances which have been reported to possess antineuritic properties when tested on pigeons, have no value as a source of water-soluble B when tested under the conditions described above, using the rat as a subject, *and involving the element of growth as an essential feature of the test.*

We were fortunate in discovering, about 2 years ago, that glacial acetic acid is an excellent solvent for water-soluble B, and by the aid of this solvent have made many preparations which are very potent in the dietary essential in question. We are employing glacial acetic acid in connection with other solvents which do not dissolve the vitamin in connection with our efforts to isolate this substance.

For many obvious reasons the use of glacial acetic acid is a fortunate one. It is cheap and readily obtainable. Its use results in a preparation which is solid and easily powdered, whereas water or alcohol yields a viscid, gelatinous, semisolid mixture containing the antineuritic factor. Acetic acid is a water-miscible solvent and easily penetrates biological material, animal or plant, wet or dry, in the form of small lumps or in powder. It has a comparatively low boiling point, 118°C., and hence

can be conveniently distilled off under slightly reduced pressure at a temperature at or below the boiling point of water without inactivating the vitamin. Since the solvent is an acid it may aid in inhibiting or preventing the oxidation of water-soluble B, which may be facilitated by stirring or exposure to air, especially at high temperatures. Many organic compounds, monosaccharides, disaccharides, phenols, etc., easily undergo oxidation in an alkaline medium but this process suffers retardation in the presence of acid. A solution of water-soluble B in glacial acetic acid should be by *a priori* reasoning very active even after long standing. This we have proved to be the case. A very unique property of glacial acetic acid from the standpoint of the isolation of vitamin in the pure state lies in its ability to precipitate carbohydrates. Still another and not unimportant consideration arguing for the use of glacial acetic acid is its miscibility in all proportions with ether—a condition which enhances the value of the latter as a means of purifying the antineuritic factor by removal by precipitation of a large amount of inactive material from the active mixture.

The following brief statement of a procedure which will be of interest to investigators in this field for separating water-soluble B from a large part of the components of a natural food, is made at this time, because if affords a new aid to inquiry in this direction. A typical test of the biological value of the preparation is also included.

1,000 gm. of ground raw navy beans and 3,000 cc. of glacial acetic acid were heated for 3 hours on the water bath. The mixture was filtered hot on two layers of cloth, the residue washed again with small quantities of 1,000 cc. of glacial acetic acid, and heated with 3,000 cc. of glacial acetic acid for another 3 hour period. The hot mixture was filtered on two layers of cloth and washed with 1,000 cc. of glacial acetic acid, using small quantities at a time. The filtrate was now passed through fluted paper. The resulting, clear, reddish brown liquid was distilled at 70°C. under reduced pressure and the residue dried with the electric fan. The final result is a solid, which readily lends itself to powdering.

27.5 gm. of solid extract prepared as described above, and corresponding to 250 gm. of navy beans, were dissolved with the aid of heat in 1,000 cc. of glacial acetic acid. This was precipi-

tated with 5 volumes of ether.   The precipitate, a solid, choco-
late-brown substance, was removed by filtration.   The filtrate
was evaporated to dryness with a blast of air from an electric
fan, and left a chocolate-colored solid residue.   The precipitate
(Preparation I) weighed 15.5 gm., and the residue (Preparation
II) from the evaporation of the filtrate weighed 12.0 gm.   Each
of these preparations was tested for water-soluble B, using young
rats as test animals.

A group of young rats weighing about 45 to 70 gm. was restricted
to the following diet, which was complete except for the absence
of water-soluble B.

	per cent
Casein ... .,................18.0	
Salt mixture (185).. 3.7	
Agar-agar 2.0	
Dextrin........ ....71.3	
Butter fat 5.0	

The casein employed had been carefully freed from almost
all traces of inorganic salts and vitamins by a method previously
described.   It consists of washing the finely ground casein in
distilled water acidified to the extent of about 0.2 per cent with
acetic acid.   During the 1st day four changes of tap water are
made.   It is then soaked over night in acidified, distilled water.
The casein is daily removed on cheese-cloth and freed from most
of the liquid, then returned to fresh distilled water and acidified
again.   This treatment is continued through 7 days.   The salts
of the crude casein are thus caused to dialyze out of the granules,
and the impurities are completely washed from the swollen gran-
ules.   Such a procedure we have found through extensive ex-
perience to result in a preparation which is free from demonstrable
amounts of any vitamin.

On the diet described small rats are able to increase in weight
in some cases for about 2 or 3 weeks.   Their growth is then sus-
pended and they gradually become attenuated of form and en-
feebled.   Many reach a stage where they toss the head backward
repeatedly, and at intervals show the signs of opisthotonos.
Finally, they may develop the symptoms of acute polyneuritis,
but some die without reaching this condition.   When it is evident
that steady physical decline has set in, the animals are ready for

the administration of any curative preparation which it is desired to test, for they never improve if confined to the experimental diet as their sole source of nutriment.

Rats fed the experimental diet for 6 weeks, and which had begun to decline, were given Preparation I, equivalent to 50 per cent of beans in the diet. This preparation is the precipitate formed by pouring ether into a glacial acetic acid solution containing the glacial acetic acid-soluble matter derived from raw navy beans. The test was negative. The animals continued to decline and died. This indicates the nearly complete absence of water-soluble B from the precipitate in question.

Rats fed the experimental diet as above described were given, after decline had set in, the material which remained in solution when a filtered acetic acid extract of raw navy beans was poured into five times its volume of ether (Preparation II). The amount administered was equivalent to 50 per cent of beans in the diet. The animals responded in a manner fully as remarkable as they would have done had a liberal amount of a natural food containing water-soluble B been made to replace half of their experimental diet.

We do not desire at this time to discuss in detail the degree of potency of this and similar preparations, since we are carrying on further studies in the direction of perfecting a method of freeing as far as possible the vitamin, water-soluble B, from contaminating substances.

CONCLUSIONS.

Glacial acetic acid is the best organic solvent which we have yet found for the extraction of water-soluble B from plant materials. The active glacial acetic acid extract can be further concentrated by the removal by precipitation with ether of a large quantity of inactive material.

BIBLIOGRAPHY.

1. McCollum, E. V., and Simmonds, N., A study of the dietary essential, water-soluble B, in relation to its solubility and stability towards reagents, *J. Biol. Chem.*, 1918, xxxiii, 55.
2. Dutcher, R. A., Vitamine studies. IV. Antineuritic properties of certain physiological stimulants, *J. Biol. Chem.*, 1919, xxxix, 63.
3. McCollum, E. V., and Koch, M., Unpublished data.
4. McCollum, E. V., and Simmonds, N., Unpublished data.

A MODIFICATION OF THE BELL-DOISY PHOSPHATE METHOD.

By A. P. BRIGGS.

(*From the Laboratories of Biological Chemistry, Washington University School of Medicine, St. Louis.*)

The colorimetric phosphate method of Bell and Doisy,[1] if followed carefully as described, gives results which check gravimetric determinations, but this method has one objection; namely, that the alkaline blue color which is used for comparison in the colorimeter fades rather rapidly. For this reason it is not advisable to read more than about two determinations against the same standard. In the first stage of color production a stable green is produced in acid solution which is proportional to the phosphorus present. This color was not used by Bell and Doisy[1] for comparison (personal communication from Dr. Doisy) because with either urines or trichloroacetic acid blood filtrates there is an occasional turbidity produced when the acid molybdate solution is added which interferes with the color comparison, due to a precipitate of undetermined nature. In attempting to overcome these difficulties, it was found by the writer that by a small modification during the trichloroacetic acid precipitation of blood or plasma, the turbidity can be avoided, thus allowing the use of the acid solutions for color comparison. When blood or plasma is diluted with 3 volumes of water and 1 volume of 20 per cent trichloroacetic acid in an Erlenmeyer flask, shaken vigorously for a few seconds, and then allowed to stand for about 10 minutes before filtering, the filtrates give with acid molybdate and hydroquinone perfectly clear green colors. The supposition is that when the blood is diluted in a volumetric flask and mixed merely by inverting a few times, a small amount of protein gets through into the filtrates and this forms a precipitate with

---

[1] Bell, R. D., and Doisy, E. A., *J. Biol. Chem.*, 1920, xliv, 55.

the molybdic acid. A parallel series of determinations on plasma filtrates showed that identical results are obtained, whether the acid green color is used for comparison or the comparisons made of the alkaline blue according to the technique of Bell and Doisy. The green color is considerably less intense, and accurate comparison is with low phosphorus plasmas difficult; but the stability of color offsets this disadvantage.

It has also been recently observed that when a little sodium sulfite is added to an acid solution containing phosphate and molybdate that the subsequent addition of hydroquinone causes the formation of a blue instead of a green color and of an intensity considerably greater than the green. This color does not depend upon reduction of the molybdic acid by SO_2 since sodium sulfite, hydroquinone, and acid molybdate solutions when mixed give no color. The use of these modifications gives a clear blue, non-fading color for comparison, the proportionality of which is exact over a wide range. The intensity of the color allows the determination of phosphates in 1 cc. of plasma. The following technique is that used for blood or plasma: A measured volume of plasma is transferred to a small Erlenmeyer flask, diluted with 3 volumes of water and 1 volume of 20 per cent trichloroacetic acid. The flask is stoppered with the thumb, shaken vigorously for a few seconds, and after standing about 10 minutes, the contents are transferred to a dry ashless filter. The filter funnels rest in long Pyrex test-tubes and are covered by watch-glasses to prevent loss by evaporation. For the determination, transfer 5 cc. of the filtrate, equivalent to 1 cc. of plasma, to a 10 cc. volumetric flask or a long test-tube graduated at 15 cc. For the standard, transfer 2 cc. of the diluted phosphate solution, to a similar flask or tube. To each then add 2 cc. of the molybdate solution, 1 cc. of the sodium sulfite solution, and 1 cc. of the hydroquinone solution, and dilute with water to the mark. Allow them to stand about 30 minutes for color production and compare in the colorimeter.[2]

    [2] It is not necessary to add trichloroacetic acid to the standard to balance that of the filtrate. It is necessary, however, to have the acidity within certain limits for color production. Sufficient acid is provided by 2 cc. of the molybdate reagent for the formation of ammonium phosphomolybdate and its subsequent reduction; on the other hand if the total acidity after addition of all reagents is more than about 2 N then no color will be obtained.

Solutions Used.

Standard Phosphate Solution for Urine.—This solution contains 0.4394 gm. of dry KH_2PO_4 per liter. 1 cc. is equivalent to 0.1 mg. of phosphorus. Chloroform is added as preservative.

Standard Phosphate Solution for Blood.—25 cc. of urine phosphorus standard is diluted to 200 cc. and preserved with chloroform. 2 cc. of this solution are equivalent to 0.025 mg. of phosphorus.

Molybdate Solution.—25 gm. of ammonium molybdate are dissolved in 300 cc. of water. To this are added 200 cc. of water containing 75 cc. of concentrated H_2SO_4.

Hydroquinone Solution.—0.5 gm. of hydroquinone is dissolved in 100 cc. of water and a drop of concentrated H_2SO_4 added to retard oxidation. 1 cc. of this solution provides an abundant excess even in the determinations on urines high in phosphorus.

Sulfite Solution.—This solution contains 20 per cent sodium sulfite. It should be kept well stoppered or made fresh.

The following test was carried out to test the proportionality between the phosphorus present and the color produced by the new technique. Amounts of the standard phosphate solution varying from 7 to 25 cc. were transferred to 100 cc. volumetric flasks. To each were added in succession 5 cc. of the molybdate solution, 1 cc. of the sulfite solution, and 1 cc. of the hydroquinone solution. They were then diluted with water up to the mark, mixed by inverting a few times, and allowed to stand about an hour for color production. Each was then compared with the one containing 15 cc. The average of several readings of each solution showed a perfect proportionality over this range.

The modified technique was compared with the Bell-Doisy procedure on ten rabbit bloods with the results given in Table I.

The whole rabbit blood was taken because of the inconsistencies recently reported by Myers and Shevky[3] with the Bell-Doisy technique on "many" rabbit bloods. All of these filtrates gave perfectly clear blue colors by the modified technique and clear bluish green colors after the addition of molybdate and hydroquinone by the Bell-Doisy technique.

The modified technique is also applicable to urines. Comparison with the Bell-Doisy technique was made on a few urines

[3] Myers, B. A., and Shevky, M. C., *J. Lab. and Clin. Med.*, 1921-22, vii, 176.

according to the following procedure: Take 1 to 5 cc. of acidified urine or an amount equivalent to about 0.5 mg. of P, in a 100 cc. volumetric flask. In a similar flask, take 5 cc. of the urine P standard. Dilute each with water up to about 80 cc. Then add to each 5 cc. of the molybdate solution, 1 cc. of the sulfite solution,

TABLE I.

Comparison of the Bell-Doisy Method with Modified Technique on Ten Rabbit Bloods.

Blood	Bell-Doisy method.	Author's modification.
	mg. per 100 cc.	*mg. per 100 cc.*
1	8.19	8.57
2	5.93	6.22
3	9.90	9.88
4	10.20	10.01
5	8.19	8.26
6	8.76	8.57
7	7.50	7.50
8	10.05	10.07
9	8.51	8.66
10	8.51	8.63

TABLE II.

Analysis of Three Urines by the Two Methods.

Urine.	Bell-Doisy method.	Author's modification.
	gm. per liter	*gm. per liter*
1	1.21	1.14
2	1.28	1.32
3*	1.54	1.56

* Urine 3 gave a precipitate of undetermined nature which was removed by centrifugation while the color was developing.

and 1 cc. of the hydroquinone solution. Dilute each with water up to the mark and allow to stand about $\frac{1}{2}$ hour for color production. The results are given in Table II.

Addendum.—After this paper had been sent to press, it was shown by Denis[4] that oxalates and citrates interfered with the formation of the blue color of the Bell-Doisy method. It has been found here, however, that such amounts of citrates or oxalates as are used to prevent clotting of blood do not interfere with the formation of the acid blue and so no error is introduced in the determination by the modified technique.

[4] Denis, W., and von Meysenbug, L., *J. Biol. Chem.*, 1922, lii, 1.

A COLORIMETRIC DETERMINATION OF BLOOD CHLORIDES.

By M. L. ISAACS.

(*From the Department of Biochemistry, University of Cincinnati, Cincinnati.*)

The following colorimetric method makes use of the Folin and Wu filtrate, is rapid, and employs a permanent standard.

Principle.

Since silver chloride is about one-thirtieth as soluble as red silver chromate, it follows that silver chromate will dissolve in a solution of chlorides; the silver being reprecipitated as chloride, while a certain amount of yellow chromate goes into solution according to the following equation:

$$2NaCl + Ag_2CrO_4 \rightarrow 2AgCl + Na_2CrO_4$$

The reaction is familiar to the analytical chemist in the Mohr titration of chlorides. Silver chromate imparts only a very slight color to distilled water.

Reagents.

1. Silver chromate. (Red modification.) This is best prepared by adding slowly 200 cc. of a 5.5 per cent solution of potassium chromate to 100 cc. of a boiling solution of silver nitrate (10 per cent). The silver chromate settles out rapidly. Drops of the chromate solution are added until there is a slight excess of chromate, which gives the solution a yellow color. After cooling, the silver chromate is thoroughly washed with distilled water and finally air-dried on a Buchner funnel.

2. Magnesium carbonate.

3. Ammonium hydroxide, 2 per cent.

Procedure.

10 cc. of the Folin and Wu filtrate are pipetted into a small conical centrifuge tube (which has been previously cleaned with

17

warm chromic acid solution). A pinch of magnesium carbonate is added to insure neutrality of the liquid. The contents of the tube are stirred with a thin glass rod. A small quantity (about 0.05 gm.) of silver chromate is introduced and thoroughly stirred into the solution. If all the red particles disappear more chromate must be added. After washing off the stirring rod into the tube, the tube is centrifuged for 2 minutes. The contents are then decanted through a small filter, into a 25 cc. volumetric flask, great care being taken not to disturb the residue at the bottom of the tube. After the addition of 10 cc. of water to the tube, the centrifuging is repeated for 5 minutes. The contents of the tube are then filtered into the volumetric flask. The solution has a slight turbidity which is cleared up by the addition of 1 cc. of a 2 per cent ammonium hydroxide solution. Enough water is added to bring the solution to the mark. After mixing, comparison is made with a standard potassium chromate solution containing 0.4 gm. of the salt per liter. The value of this standard may be found by employing 5 cc. of a 0.02 x solution of sodium chloride in place of 10 cc. of blood filtrate.

Since yellows are difficult to match, the colors can be viewed through a blue glass, as suggested by Michaelis.[1]

With the chromate solution used above, the chromate being 99.4 per cent pure, with the colorimeter standard at 20, the following formula applies:

$$\frac{11.730}{\text{Unknown reading}} = \text{mg. sodium chloride per 100 cc. blood}$$

In the following blood filtrates the method of Whitehorn[2] was used as a rapid check.

Sample.	Whitehorn's method.	Colorimetric method.
1	484	481
2	490	502
3	517	516
	517	520
4	492	504
5	477	489
6	503	504

[1] Michaelis, L., *Deutsch. med. Woch.*, 1921, xlvii, 465.

[2] Whitehorn, J. C., *J. Biol. Chem.*, 1920-21, xlv, 449.

Using 5 cc. of filtrate good results were obtained, but the colors were hard to match.

Mention should be made of the possible effects of other salts in the filtrate. Silver phosphate is slightly less soluble than silver chromate and it would be expected that silver chromate would dissolve in a solution containing phosphate. This does take place, but in very dilute solutions such as the blood filtrate the color develops very slowly. Furthermore, the phosphates of the blood are probably acid phosphates which do not react with the chromate. This was shown by adding 0.0010 gm. of monosodium hydrogen phosphate to 5 cc. of blood filtrate, an amount which would correspond to an extreme case of phosphate retention. As a check, 5 cc. of the same filtrate were taken and both filtrates were treated with magnesium carbonate and silver chromate. No difference of color could be observed. Other silver salts which are less soluble than the chromate are either absent or present in negligible quantities in the filtrate.

Using the principle of differential solubilities it is hoped that methods can be worked out for calcium, potassium, magnesium, phosphates, and other ions.

DIETARY FACTORS INFLUENCING CALCIUM ASSIMILATION.

II. THE COMPARATIVE EFFICIENCY OF DRY AND GREEN ALFALFA IN MAINTAINING CALCIUM AND PHOSPHORUS EQUILIBRIUM IN MILKING COWS.*

By E. B. HART, H. STEENBOCK, and C. A. HOPPERT,

(*From the Department of Agricultural Chemistry, University of Wisconsin, Madison.*)

and G. C. HUMPHREY.

(*From the Department of Animal Husbandry, University of Wisconsin, Madison.*)

The fact that green plant tissue (1) contains a more liberal quantity of a vitamine favoring calcium assimilation than does dried plant tissue led us to a study of this problem in its relation to the calcium and phosphorus metabolism of milking cows. We had in earlier work (2) observed very marked negative calcium balances in a liberally milking cow receiving a dry cereal straw as her roughage. In this case the daily calcium oxide intake was 25 gm. with a daily milk yield of 30 to 38 pounds.

In extensive experiments involving a number of years of work, Forbes (3) and his associates have observed negative calcium balances with milking cows receiving the best of dried forage in respect to its calcium content; namely, alfalfa or clover hay. In some of these cases the daily calcium oxide intake was as high as 175 gm. with a daily milk production of 51 pounds and a utilization of but 40 gm. of calcium oxide for this milk production; yet a negative calcium balance was reported.

Meigs, Blatherwick, and Cary (4) have presented data indicating that a dry, pregnant cow is probably not assimilating sufficient

---

calcium from a calcium-rich ration such as alfalfa hay, corn silage, and a grain mixture for a positive calcium balance, but is actually transferring calcium salts from her skeleton for fetal skeleton-building. Meigs and his associates are inclined to interpret the observed negative calcium balances as only temporary and merely induced by the nervous disturbances of the animal incidental to the collection of the excreta and general interference with the freedom of the animal.

Of the above observations those of Forbes were outstanding and his work made it appear probable that a liberally milking cow fed the richest available carrier of calcium as forage, such as dry alfalfa hay, would nevertheless be compelled to draw on her own mineral reserves for maintenance of milk production. We accepted this point of view and formulated the hypothesis that the negative calcium balance of a milking cow receiving dry alfalfa hay would be turned to a positive one if the alfalfa were fed *fresh* and *green*. In fact, it appeared probable to us that it must be during the period of green pasturage that the depleted mineral reserves of dairy cattle, incident to a long winter milking period, would be replenished. True, the factor of the nature of the green grasses would be important because it would be entirely possible that if the grasses were of those varieties naturally low in lime and in addition had grown on an acid soil, the factor of mere greenness and consequently richness in a vitamine assisting calcium assimilation would not be sufficient to establish positive calcium balances with liberally milking animals. Further, it appeared to us unbelievable that the dairy cattle of our country were in constant negative calcium balance, or else a low average milk production and an early termination of the life of the individual as a milk producer must follow, an assumption that does not appear to be supported by facts. However, it is very probable that a condition of negative calcium balance in milking cows does widely prevail especially in the winter time and in regions where low calcium-carrying roughages are used—a condition that must have an important bearing on the yield of milk, resistance to disease, and reproduction (5).

Our plan was to feed liberally milking cows dried alfalfa hay plus silage and a grain mixture over a period of time sufficient in length to establish the assumed negative calcium balance and

then to replace the dried alfalfa hay with fresh, green alfalfa in an amount equivalent in dry matter to the dry hay. The results we secured in our first experiments with *dry* alfalfa hay support the view that it is possible to maintain calcium equilibrium in high milking cows with such a roughage. These particular results are contrary to the findings of Forbes and his associates as well as contrary to results we, ourselves, have secured in later experiments with another alfalfa hay (data to be published later). Equilibrium or positive calcium balances were obtained with this *dry* alfalfa hay as well as with the *green* and succulent alfalfa.

EXPERIMENTAL.

For this work three pure-bred Holstein cows were used. No. 1 weighed 1,372 to 1,468 pounds; No. 2, 1,093 to 1,107 pounds; and No. 3, 1,284 to 1,293 pounds, at the beginning and end of the experiment which ran from May 11th to July 5th, 1921. No. 1 had freshened in December, 1920; No. 2 in October, 1920; and No. 3 in May, 1920.

The animals were confined to metabolism stalls with quantitative collection of the excreta and milk. The collection of the excreta, as in all of our experiments of this character, was always done by men working in 8 hour shifts.

No. 1 was with calf and due to freshen September, 1921. She had been on a poor mixed hay (low in calcium) before being placed in the experiment and it is apparent from the data in Table II that she was in condition for a more pronounced storage of calcium and phosphorus than either of the other two animals. Nos. 2 and 3 had been receiving dry alfalfa hay for 20 weeks prior to being taken into the experiment and did not show as large a storage as did No. 1. Cows 2 and 3 were not with calf.

Calcium determinations were made on all the feeds, milk, and excreta by the McCrudden method. Phosphorus was determined in the feeds, milk, and feces after ashing in the presence of magnesium nitrate. In the urine phosphorus was determined by the Neumann method, that is, after oxidation with nitric acid in the presence of sulfuric acid.

The feeds used in the first period of 4 weeks duration were corn silage, *dry* alfalfa hay, and a grain mixture made up of 60 parts of yellow corn, 25 parts of wheat bran, and 15 parts of oil meal. The alfalfa hay was of good quality, second cutting, *cured under*

caps, and grown on a southern Wisconsin farm. It retained a fairly bright green color and was judged as first quality. The alfalfa hay was chopped before feeding. For $3\frac{1}{2}$ pounds of milk production 1 pound of the grain mixture was allowed. The daily ration consisted of 30 pounds of corn silage, 10 pounds of dry alfalfa hay, and an amount of the grain mixture proportional to the milk produced. On this basis No. 1 received 7 pounds of grain mixture daily, No. 2 received 13 pounds daily, and No. 3 received 9 pounds daily during the entire 8 weeks of the experiment and including both the dry and green alfalfa feeding periods. It should be noted that the daily allowance of silage and alfalfa hay was constant for the three animals during the entire time of the experiment.

TABLE I.

Calcium Oxide and Phosphorus Pentoxide Content of Feeds Used.

Material.	CaO	P_2O_5	Remarks.
	per cent	*per cent*	
Alfalfa hay........	1 82	0 73	Used in first period.
Corn meal.........	0 027	0 59	
Wheat bran.......	0 170	2.68	
Oil meal..........	0 557	2 31	
Corn silage.......	0 50	0 57	Air-dried condition.
Green alfalfa......	1 42–2.05	0.57–0.73	Analysis on air-dried material.

Following the 4 weeks of *dry* alfalfa hay feeding was a period of 4 weeks during which *fresh, green* alfalfa displaced the dry hay. The rest of the ration remained the same. The green alfalfa was cut daily from a field that had already been cut once and was for the most part in prime condition.

Water determinations were made daily on the green material in order to keep the dry matter of the ration derived from the green alfalfa as constant as possible and equivalent to the 10 pounds of air-dried hay fed daily in the first period. Further, samples of the green alfalfa were taken daily for calcium and phosphorus determinations. All the animals received distilled water. Common salt was fed *ad libitum*.

In Table I the calcium and phosphorus content of the feeds used are given. In the case of the corn silage, the percentages are as found on the air-dried material. No constant figures can

be given for the green alfalfa hay as it varied slightly from day to day; the CaO content of the air-dried material derived from the

TABLE II

Record of Calcium Balance of Animal 1.

Period	CaO in feces.	CaO in urine	CaO in milk	Total CaO excreted	Total CaO intake	Balance per week.	Balance per day.	Milk per week.
	gm.	*gm*	*gm*	*gm*	*gm*	*gm*	*gm.*	*lb.*
Dry hay period.								
May 11–17	451 54	1 76	178 20	631 50	784 94	+153 40	+21 91	160
" 18–24	524 81	1 21	172 07	698 09	784 94	+ 86 85	+12 41	151
" 25–31	534 32	1 32	156 06	691 70	773 23	+ 81 53	+11 36	137
June 1–7	537 08	0 94	136 37	674 39	773 23	+ 98 84	+14 12	122
Green hay period.								
June 8–14	563 97	1 09	110 23	675 29	768 81	+ 93 52	+13 36	98
" 15–21	523 58	2 81	75 06	601 45	802 46	+201 01	+28 71	66
" 22–28	613 42	2 22	65 28	680 92	1,025 37	+344 45	+49 21	64
" 29–July 5	441 57	1 55	60 66	503 78	808 12	+304 34	+43 48	63

Record of Phosphorus Balance of Animal 1.

Period	P_2O_5 in feces	P_2O_5 in urine	P_2O_5 in milk	Total P_2O_5 excreted.	Total P_2O_5 intake	Balance per week.	Balance per day.
	gm.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
Dry hay period.							
May 11–17	409 42	15 00	182 41	606 83	747 55	+140 72	+20 10
" 18–24	397 15	17 35	170 00	584 50	747 55	+163 05	+23 29
" 25–31	436 28	8 62	153 58	598 48	734 20	+135 72	+19 39
June 1–7	527 18	11 36	134 70	673 27	734 20	+ 60 96	+ 8 71
Green hay period.							
June 8–14	528 56	2 71	100 35	631 32	719 62	+ 88 00	+12 57
" 15–21	484 70	5 63	72 66	562 99	760 09	+197 10	+28 14
" 22–28	527 94	4 96	71 16	604 06	792 41	+188 35	+26 91
" 29–July 5	443 72	4 66	72 91	521 29	749 90	+228 61	+32 66

green alfalfa varied from 1.42 to 2.05 per cent and the P_2O_5 from 0.57 to 0.73 per cent.

In Tables II, III, and IV are recorded the data on the income and outgo of calcium and phosphorus for the three animals, re-

spectively; in addition there is added a column of the milk yield for periods of 7 days. It should be noted that there was a decline in milk yield in the case of Animals 2 and 3 after the 5th week of

TABLE III

Record of Calcium Balance of Animal 2.

Period	CaO in feces	CaO in urine	CaO in milk.	Total CaO excreted.	Total CaO intake.	Balance per week.	Balance per day.	Milk per week.
	gm.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*lb.*
May 11–17	619 85	0 60	211 89	832 34	812 01	−20 33	− 2 90	288
" 18–24	543 68	0 61	223 58	767 87	812 01	+44 14	+6 30	288
" 25–31	571 20	1 84	219 58	792 62	800 03	+ 7.68	+1.10	284
June 1–7.....	541 82	0 85	215 24	857 91	800 03	+42 12	+6 02	285

Dry hay period. (applies to above section)

Period	CaO in feces	CaO in urine	CaO in milk.	Total CaO excreted.	Total CaO intake.	Balance per week.	Balance per day.	Milk per week.
June 8–14 ..	523 80	0 61	197 15	721 56	795 88	+ 74 32	+10 62	261
" 15–21	611 14	0 67	181 30	793 11	856 69	+ 63 58	+ 9.08	237
" 22–28 .	690 12	0 51	206 58	897 21	1,055.54	+158.33	+22.62	246
" 29–July 5	661 42	0 64	188 98	851 04	882 10	+ 31.06	+ 4.44	227

Green hay period. (applies to above section)

Record of Phosphorus Balance of Animal 2.

Period.	P_2O_5 in feces.	P_2O_5 in urine.	P_2O_5 in milk.	Total P_2O_5 excreted.	Total P_2O_5 intake.	Balance per week.	Balance per day.
	gm.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
May 11–17.........	556 42	21 05	239.36	816 83	997.66	+180 83	+25 83
" 18–24	645 09	19 08	235.35	899.52	997.66	+ 98 14	+14 02
" 25–31.........	625 46	25 02	236 36	886.84	984 31	+ 97.47	+13.92
June 1–7.........	722 42	21 33	239 87	983 62	984.31	+ 0.69	+ 0.10

Dry hay period. (applies to above section)

Period.	P_2O_5 in feces.	P_2O_5 in urine.	P_2O_5 in milk.	Total P_2O_5 excreted.	Total P_2O_5 intake.	Balance per week.	Balance per day.
June 8–14	688 50	11 21	217 35	917 06	969.73	+ 52 67	+ 7.52
" 15–21	780 45	6 65	187.75	974 85	1,023.12	+ 48 27	+ 6 89
" 22–28.........	728 46	4 33	206 46	939 25	1,043 61	+104 36	+14 91
" 29–July 5 ...	704 09	6 42	178 76	889 27	1,018 47	+129.20	+18 47

Green hay period. (applies to above section)

observation, and in the case of Animal 1 somewhat earlier. This decline we attribute to the very hot weather prevailing at that time and trouble with flies.

TABLE IV.

Record of Calcium Balance of Animal 3.

Period.	CaO in feces.	CaO in urine	CaO in milk.	Total CaO excreted	Total CaO intake.	Balance per week.	Balance per day.	Milk per week.
	gm.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*lb.*

Dry hay period.

Period.	CaO in feces.	CaO in urine	CaO in milk.	Total CaO excreted	Total CaO intake.	Balance per week.	Balance per day.	Milk per week.
May 11–17......	680.44	0.64	136.32	817.40	793.97	−23.43	−3.35	179
" 18–24......	645.89	0.78	140.31	786.98	793.97	+ 6.99	+0.99	175
" 25–31......	629.56	0.58	132.36	762.50	782.26	+19.76	+2.82	171
June 1–7.......	598.60	0.47	130.23	729.30	782.26	+52.96	+7.56	172

Green hay period.

Period.	CaO in feces.	CaO in urine	CaO in milk.	Total CaO excreted	Total CaO intake.	Balance per week.	Balance per day.	Milk per week.
June 8–14......	594.60	0.48	126.28	721.36	777.84	+ 56.48	+ 8.07	169
" 15–21......	602.64	0.92	107.81	711.37	838.65	+127.28	+18.18	139
" 22–28	708.78	0.67	113.79	823.24	1,037.50	+214.26	+30.61	128
" 29–July 5..	681.72	0.74	102.17	784.63	868.33	+ 83.70	+11.96	128

Record of Phosphorus Balance of Animal 3.

Period.	P₂O₅ in feces.	P₂O₅ in urine	P₂O₅ in milk.	Total P₂O₅ excreted	Total P₂O₅ intake	Balance per week.	Balance per day.

Period.	P_2O_5 in feces.	P_2O_5 in urine	P_2O_5 in milk.	Total P_2O_5 excreted	Total P_2O_5 intake	Balance per week.	Balance per day.

Dry hay period.

Period.	P_2O_5 in feces.	P_2O_5 in urine	P_2O_5 in milk.	Total P_2O_5 excreted	Total P_2O_5 intake	Balance per week.	Balance per day.
	gm.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
May 11–17.........	654.71	22.33	166.46	843.50	823.51	− 19.99	− 2.85
" 18–24.........	507.12	12.56	152.23	671.91	823.51	+141.60	+20.23
" 25–31.........	629.57	17.68	149.50	796.75	810.16	+ 13.41	+ 1.91
June 1–7...........	646.07	10.55	147.49	804.11	810.16	+ 6.05	+ 0.86

Green hay period.

Period.	P_2O_5 in feces.	P_2O_5 in urine	P_2O_5 in milk.	Total P_2O_5 excreted	Total P_2O_5 intake	Balance per week.	Balance per day.
June 8–14.........	626.18	3.58	138.60	768.36	795.58	+ 27.22	+ 3.89
" 15–21.........	622.17	6.13	112.85	741.15	848.97	+107.82	+15.40
" 22–28.........	672.28	5.78	114.98	793.04	869.46	+ 76.42	+10.92
" 29–July 5.....	653.94	6.20	110.35	770.49	846.03	+ 75.54	+10.79

DISCUSSION.

It can be seen from the data in Table II that Animal 1 was in positive calcium and phosphorus balances on both the dry and the green alfalfa, the average daily storage of calcium being, respectively, 14.95 gm. on the dry alfalfa hay and 33.49 gm. on the green alfalfa. This larger storage of calcium on the green alfalfa we would interpret as being in harmony with previous observa-

tions that green plant tissue contains more than dried plant tissue
of some vitamine favoring calcium assimilation.

Animals 2 and 3 were also in positive calcium and phosphorus
balances in both periods of observation, but not to the same de-
gree as No. 1.   This difference is to be explained by the fact that
No. 1 had been receiving poor hay (low in calcium) previous to
being put on the experiment, and consequently was in a more
depleted condition with respect to the minerals (calcium and phos-
phorus) than Nos. 2 and 3, which had received alfalfa hay for 20
weeks previous to being placed in this experiment.   In agreement
with the results secured with No. 1 there was a greater tendency
on the part of these animals to store calcium during the green
alfalfa period than during the dry alfalfa period by both Animals
2 and 3.   No. 2 showed a positive daily average calcium balance
of but 2.6 gm. during the dry alfalfa period but this was increased
to 11.66 gm. in the green alfalfa period.   No. 3 showed an average
positive daily calcium balance of 2.00 gm. during the dry alfalfa
period and 17.20 gm. during the green alfalfa period.   No. 2
was an especially heavy milker—yielding over 40 pounds of milk
per day.

The more pronounced calcium storage observed during the
green alfalfa period as compared with the *dry* alfalfa period is not
to be explained as due to differences in calcium intake alone.
While the calcium intake during the green alfalfa period was in
some cases higher than during the dry alfalfa period, yet the re-
tention of calcium was so much greater in the former period as to
exclude the slight differences in the quantity of calcium ingested
as the sole determining factor in the results.

Irrelevant to the direct purpose of this paper but well worth
recording was the fact that the milk of No. 2 during the feeding of
dry alfalfa hay and also previous thereto, but only while receiving
dry hay, was coagulable by heat at 136°, the temperature of a
boiling xylene bath.   This coagulability could be prevented by
the addition of calcium salts (6).   When this animal was changed
to the green alfalfa hay, the milk still retained the property of
coagulability by heat at 136°C., but instead of being corrected by
the addition of calcium salts, the coagulation point was lowered
by such additions and was corrected for by the addition of citrates.

Why these data on calcium balances are at variance with those reported by Forbes and with later results secured by ourselves it is difficult to see. The only suggestion as an explanation that we can offer at the present time is the possibility of a difference in the character of the dry alfalfa hay and silage fed. The dry alfalfa hay which we used may possibly have been richer in the vitamine, assisting calcium assimilation, than the alfalfa used by Forbes in his experiments and the alfalfa used by us in our second series of experiments to be reported upon in a later publication.

With the corn silage the situation may have been similar. The maturity of the corn and the process of ensilaging, like the curing of the alfalfa are variable factors which may modify the nutritive value of the product.

SUMMARY.

1. Liberally milking cows were maintained in positive calcium balance by *dry* alfalfa hay used as the principal roughage and main source of calcium when supplemented with corn silage and a grain mixture. The hay consumption was 10 pounds per individual per day. It was of excellent quality, having been cured under caps.

2. On *fresh green* alfalfa more liberal storage of calcium was observed with these animals than on dry alfalfa hay.

3. With positive calcium balances there were also positive phosphorus balances with the three animals under observation.

4. These results, in reference to calcium equilibrium in milking cows, do not appear to be in harmony with the findings of Forbes and his associates. We have additional data, however, secured recently and to be published in a later paper, but obtained with another alfalfa hay, which also gave us negative calcium balances.

Apparently the question whether positive or negative calcium balances will prevail in liberally milking cows through the use of such an efficient carrier of calcium as alfalfa hay is determined by the quality of the alfalfa hay used. By the term quality, used in this connection, we mean the relative degree of destruction in the curing processes of the unknown factors affecting calcium assimilation.

30   Calcium Assimilation.   II

BIBLIOGRAPHY.

1. Steenbock, H., and Hart, E. B., *J. Biol. Chem.*, 1913, xiv, 59; Hart, E. B., Steenbock, H., and Hoppert, C. A., *J. Biol. Chem.*, 1921, xlviii, 33.
2. Hart, E. B., McCollum, E. V., and Humphrey, G. C., *Wisconsin Exp. Station, Research Bull. 5,* 1908.
3. Forbes, E. B., Beegle, F. M., Fritz, C. M., Morgan, L. E., and Rhue, S. N., *Ohio Agric. Exp. Station, Bull. 295,* 1916; *Ohio Agric. Exp. Station, Bull. 308,* 1917. Forbes, E. B., Halverson, J. O., and Morgan, L. E., *Ohio Agric. Exp. Station, Bull. 330,* 1918.
4. Meigs, E. B., Blatherwick, N. R., and Cary, C. A., *J. Biol. Chem.,* 1919, xl, 469.
5. Hart, E. B., Steenbock, H., and Humphrey, G. C., *Wisconsin Exp. Station, Research Bull. 49,* 1920.
6. Sommer, H. H., and Hart, E. B., *J. Biol. Chem.,* 1919, xl, 137.

A METHOD FOR THE PREPARATION OF CRYSTALLINE OXYHEMOGLOBIN.

By MICHAEL HEIDELBERGER.

(*From the Hospital of The Rockefeller Institute for Medical Research.*)

Available methods for the preparation of crystalline oxyhemoglobin may be divided into two groups, in the first of which alcohol or ether, or both, are used as aids to crystallization, and in the second of which the use of these solvents is avoided. Possible objections to the use of alcohol were early pointed out by Hüfner (1), and Mayet (2) emphasized the advantages of "benzine" over ether. Although these workers, as well as Bohr (3) and Torup (4), made preparations in which the use of either or both of these substances was avoided, the first systematic attempt to work out a method without their aid seems to have been the recent one of Dudley and Evans (5). Their procedure involves pressure dialysis of the washed red cells of horse blood, crystallization of the oxyhemoglobin in the dialysate by oxidation, and recrystallization of the product from water by reduction *in vacuo* at 37° and subsequent oxidation. Data as to the yield and purity of the product are not given.

In the hope of obtaining fairly large amounts of oxyhemoglobin in the highest possible state of purity, the Dudley and Evans method was first employed, using a modification suggested by Adolph and Ferry (6); namely, final dialysis against water saturated with carbon dioxide. The oxyhemoglobin obtained in this way crystallized as large plates, instead of the needles reported by Dudley and Evans. Furthermore, the insolubility of the plates and their consequent resistance to reduction not only justified the belief that isoelectric oxyhemoglobin was being dealt with, and that Dudley and Evans were probably working with a more soluble salt, but rendered the use of the method impossible for the purpose in view, and it was abandoned in favor of a different principle.

The method now proposed depends upon observations that suspensions of washed dog or horse red cells crystallize rapidly and almost completely in the presence of toluene when saturated with carbon dioxide and oxygen, and that the resulting oxyhemoglobin may be recrystallized by solution with the aid of sodium carbonate and reprecipitated with carbon dioxide.

The use of toluene was found to hasten markedly the crystallization of the oxyhemoglobin of the corpuscles owing to its disintegrating effect on the cells themselves. While its hemolytic action is slower than that of ether, its use obviates the chief disadvantages of the latter; namely, solubility in water, and the presence of peroxides and other reactive substances which may alter oxyhemoglobin. The carbon dioxide shifts the reaction in the acid direction past the isoelectric point of oxyhemoglobin, so that the crystals obtained are oxyhemoglobin uncombined with alkali. By thus promoting the crystallization of the oxyhemoglobin the acidification also aids in the original disintegration of the corpuscles. Saturation with pure carbon dioxide would, however, drive oxygen out of the solution and change the oxyhemoglobin to the reduced form, which is too soluble to crystallize readily. In order to obviate this difficulty 1 part of oxygen was mixed in a cylinder with 4 parts of carbon dioxide for the saturation. Such a mixture may be passed through oxyhemoglobin solutions indefinitely without reduction.

Removal of the salts is accomplished by the simplified form of pressure dialysis[1] suggested by Adair, Barcroft, and Bock (7), *after* the desired number of recrystallizations has been carried out. Two recrystallizations have been deemed sufficient in this laboratory, but for many purposes the oxyhemoglobin will undoubtedly be found pure enough after the first recrystallization. On the other hand, the losses involved in each recrystallization, while appreciable, are not sufficiently large to preclude three or even four recrystallizations.

Three precautions have been found essential: (*a*) All operations are carried out in the cold, centrifugation being a possible exception if a centrifuge in a cold room is not available. (*b*) The oxyhemoglobin is not allowed to become dry, owing to the resultant change, noted by Bohr (8), into a modification in which the

---

[1] Except that the membranes are not sterilized.

TABLE I.

Preparation No.	Volume of blood.	Total oxyhemoglobin content.	Preliminary separation in centrifuge.	First recrystallization.			Second recrystallization.			Yield.		Oxygen capacity.	Conductivity at 25° of saturated aqueous solution*	Concentration of saturated aqueous solution.
				Volume of H₂O.	Total volume of suspension.	x Na_2CO_3	Volume of H₂O.	Total volume.	x Na_2CO_3					
	cc.	gm.		cc.	cc.	cc.	cc.	cc.	cc.	gm.	per cent	per cent	mhos.	per cent
Dog 8.	150	29.4	+	50	85	10–11	30	65	8.5–9	7.8	26.5	96.3	6.6×10^{-5}	4.3
" 9.	295	55.5	–	50	200	6–6.5	30	80	9–10	13.1	23.6	100.0	—	
" 10.	300	45.9	–	70	160	10–11	30	90	18–19	14.4	31.4	96.3	3.9×10^{-5}	
" 11.	465	76.3	+	100	230	18–22.5	70	170	7	29.2	38.3	99.3	5.4×10^{-5}	
" 12.	248	37.3	–	60	140	10–11	35	80		8.95	24.0	98.0	5.2×10^{-5}	3.9
Horse 6.	605	72.0	–	40	210	15	35	100	5–6	18.75	26.0	97.7	6.6×10^{-5}	2.7

* For the preparation of the solutions for conductivity determinations, see p. 38.

oxygen is not reactive. (c) During the various manipulations on the acid side of the isoelectric point, before the final dialysis, care is taken to have an excess of carbon dioxide constantly present. If the carbon dioxide tension is permitted to fall, part of the oxyhemoglobin is redissolved as alkali salt.

The purity of the oxyhemoglobin obtained by the present method has been controlled by a determination of the ratio of the oxyhemoglobin present, as determined by Van Slyke and Stadie's procedure (9), to the total hemoglobin pigments present, determined as cyanhemoglobin by Stadie's method (10). As will be seen in Table I, preparations of 96 to 100 per cent of the theoretical oxygen capacity were obtained. The relative freedom of the product from salts was controlled by conductivity measurements of saturated aqueous solutions, the values obtained being also given in the table.

EXPERIMENTAL.

Oxalated or defibrinated dog or horse blood of known oxyhemoglobin content is centrifuged and the plasma or serum and the layer of white cells are removed. The red cells are then washed three times with chilled 0.85 per cent sodium chloride solution, after which the supernatant liquid usually gives at most only a faint haze when a test portion is boiled. The cells are then rinsed into a flask with a few cubic centimeters of water. The vessel is cooled in ice water, and a steady stream of a mixture of 4 parts of carbon dioxide to 1 part of oxygen passed in. Toluene is, meanwhile, added in amount equal to about one-seventh of the volume of corpuscles, and the mixture is stirred with the gas inlet tube until it becomes pasty. Passage of the gas is continued for a few minutes, with vigorous stirring, after which the flask is stoppered tightly with a rubber stopper and allowed to stand over night in the ice box. This is often long enough to complete the process of disintegration of the cells and crystallization of the oxyhemoglobin, but if many intact cells are still to be seen under the microscope the treatment with carbon dioxide and oxygen is repeated and the flask allowed to stand a day or two longer.

The consistency of the resulting mixture depends somewhat upon the extent to which the red cells have been packed in the centrifuge and upon other factors which have not been determined.

If the mixture is sufficiently thin it may be centrifuged with advantage in chilled tubes in a cold room, separating into an upper layer of toluene and cell fragments, an intermediate layer of clear solution, and a lower layer of oxyhemoglobin crystals. The two upper layers are poured off and the crystals drained in the ice box on a chilled porous plate, the surface layer being renewed constantly as it dries out, in order to avoid possible conversion of the oxyhemoglobin into a form in which the oxygen is less reactive. During this process a slow stream of carbon dioxide should be directed over the surface of the plate, otherwise a portion of the oxyhemoglobin will redissolve as carbon dioxide evaporates from the mixture. When drainage is as complete as possible, the oxyhemoglobin is scraped into a chilled mortar and ground to a smooth paste with sufficient ice-cold water to bring the final volume up to three to three and a half times (in cubic centimeters) the weight in grams of oxyhemoglobin present in the original blood.

In case the crude mixture of crystals, toluene, and cell fragments is too thick to permit centrifugation, the entire mass is transferred to a porous plate, using the same precautions as given above. Under these conditions the process of drainage takes much longer and cannot be carried to completion owing to the emulsion formed by the toluene. On the other hand, the product, being less compact, is easier to grind to a smooth paste with water, and the toluene and cell fragments may be removed during the first recrystallization. The final volume in this case should be kept as close as possible to that given above.[2]

The thin paste of crude oxyhemoglobin is transferred to a beaker, set in ice water, and titrated to minimum turbidity with normal sodium carbonate solution. During the addition of carbonate the mixture is stirred thoroughly, and any lumps which may remain are disintegrated. The amount of sodium carbonate necessary is greatest, of course, when the crude crystals have been thoroughly drained and contain as little as possible of the bicarbonate and

[2] An alternative method, which is quite satisfactory in the case of dog blood, but is very slow in the case of horse blood, is to filter the entire mass in the ice box through silk, using as large a Buchner funnel as possible, and observing the precautions given below for filtering oxyhemoglobin suspensions.

salts of the mother liquor. In this case the final concentration of alkali added as carbonate is approximately 0.1 N. If the toluene and cell fragments have been separated previously by centrifugation and if enough water is present, a fairly clear, deep red solution will result, but if too little water is used a crystalline precipitate of what appears to be sodium oxyhemoglobinate will remain. In this case, and also in the case in which the toluene and cell fragments are still present, the carbonate solution is added to the point of minimum turbidity, after which 1 or 2 cc. more are added in order to make sure of an excess.

The solution is next centrifuged, and any toluene and cell fragments on top are sucked off through a capillary tube, a process which can generally be accomplished without appreciable loss of the actual oxyhemoglobin solution. If loss should occur, however, the mixture which has been sucked off may be whirled again and the clear oxyhemoglobin solution added to the main portion. If enough alkali has been added and there is still a crystalline deposit in the centrifuge tubes, too little water is present, and the precipitate may be dissolved in the minimum amount of water and the solution added to the main portion. This precipitate, which is usually encountered at this point only when dog blood has been used, appears to be sodium oxyhemoglobinate, for it is readily soluble in water with a bright red color, it has a characteristic crystalline form, and, finally, yields crystals characteristic of dog oxyhemoglobin when a concentrated aqueous solution is saturated with carbon dioxide-oxygen mixture and allowed to stand in the cold. Further investigation of this salt will be undertaken.

The oxyhemoglobin solution is next chilled and a stream of the carbon dioxide-oxygen mixture passed in until crystallization *begins*, after which the flask is tightly stoppered and set in the ice box. Often within a few minutes the oxyhemoglobin has set to a solid cake of long, flat, scarlet needles in the case of dog oxyhemoglobin, and dark red, glistening, broader plates, often diamond-shaped or hexagonal, in the case of horse oxyhemoglobin.

After standing over night in the ice box the crystals are sucked off on hardened paper in a Buchner funnel (the 5 inch size is adequate for the oxyhemoglobin from 300 cc. of blood). The filtration is carried out in the ice box, with a slow stream of

carbon dioxide passing into the funnel. The surface is kept moist by renewal with a spatula as it dries out, and when this is no longer possible, a few cc. of water saturated with carbon dioxide are sucked through with the same precautions, after which the filtration is stopped. The entire process usually takes less than 1 hour.

For many purposes the oxyhemoglobin is undoubtedly sufficiently pure at this point, and in one experiment which was interrupted at this stage the amount of crystalline oxyhemoglobin recovered was 46 per cent of the amount present in the original blood, as determined by the oxygen capacity.

For further purification the recrystallization process is repeated. The crystalline cake is transferred to a chilled mortar and again ground to a smooth paste with cold water. The volume of the suspension thus obtained should be about 0.7 of that employed for the first recrystallization if the toluene and cell fragments have been initially removed by centrifugation, and from 0.4 to 0.6 as large if the removal of the upper layer was accomplished during the first recrystallization. The larger fraction will, of course, be necessary when drainage of the original crystallized cell mixture on the porous plate has been most complete. The suspension of oxyhemoglobin is dissolved with normal sodium carbonate solution, centrifuged, reprecipitated with the carbon dioxide-oxygen mixture, and collected, at every step with the same precautions as in the first recrystallization.

If salt-free oxyhemoglobin is desired, the crystals are ground with the minimum amount of cold water to a paste which will just flow easily, saturated in the cold with the carbon dioxide-oxygen mixture, transferred at once to narrow collodion dialysis bags, and dialyzed under pressure in the ice box against water saturated with carbon dioxide-oxygen mixture. Dialysis for 3 or 4 days, the carbon dioxide-oxygen-saturated water and the positions of the bags being changed daily, is sufficient to bring the conductivity down to the values given in Table I. The dialysis tubes found most suitable in this laboratory were made in 50 cc. test-tubes with one of the eminently satisfactory collodion mixtures proposed by Eggerth (11), namely, a solution of 7 gm. of "Parlodion" in 60 cc. of ether, 30 cc. of alcohol, and 10 cc. of glacial acetic acid. Dialysis under pressure was accomplished by simply

closing the ends of the tubes with tightly screwed, rubber-faced screw pinch-cocks, as proposed by Adair, Barcroft, and Bock (7).

At the end of the dialysis the contents of the bags, which still retain their crystalline structure, are sucked off in the ice box on hardened paper in a Buchner funnel, using, as before, the precaution of keeping the surface layer moist. The use of carbon dioxide at this stage is unnecessary, as the oxyhemoglobin remains sparingly soluble in the absence of alkali and salts.

The conductivity values were obtained by grinding the product in a chilled mortar with ice water, centrifuging the resulting suspension, pouring off at temperatures ranging from 19 to 28°, and measuring the conductivity of the clear supernatant solution after evacuating a few times to remove any carbon dioxide present. The oxyhemoglobin content of the resulting solutions is given in Table I.

The purified oxyhemoglobin was dissolved either with the aid of sodium carbonate solution or with a sufficient excess of $N/7$ sodium hydroxide to bring the final concentration of alkali to 0.03 or 0.04 N, and the solution was filtered through a small, loose plug of washed cotton into a volumetric flask of appropriate size and made up to the mark. The yield of oxyhemoglobin was calculated from the oxygen capacity of the resulting solution, and varied between 23 and 38 per cent of the total originally present in the blood used. The purity of the product was determined by comparing the oxygen capacity with the total hemoglobin content as determined by Stadie's (10) methemoglobin method,[3] and the ratio of oxyhemoglobin to total hemoglobin pigments was found to vary between 96 and 100 per cent.[4]

The entire process of preparation of the dialyzed oxyhemoglobin can scarcely be completed in less than a week. On the other hand, it is not desirable to let preparations stand unduly long at the various stages of purification, for if the process is extended

[3] It is advisable to check up the cyanhemoglobin standard at least every 2 weeks, as the color tends to deepen, even in the ice box. The color of the chilled standard also changes with rise in temperature, so that the solution should be allowed to come to room temperature before comparisons are made.

[4] The experiments recorded in this paper were all performed before the hot weather set in. Since that time yields and oxygen capacities have occasionally dropped as much as 5 per cent below the values given above.

for much over 2 weeks there is a noticeable diminution of the
oxygen-binding power of the product. Also, when solutions
saturated with the carbon dioxide-oxygen mixture are allowed to
stand it is desirable to resaturate with the gas mixture at least
every other day in order to compensate for leakage.

The stability to be expected of the solutions of oxyhemoglobin
obtained by this method is indicated in Fig. 1, in which a steady
diminution of the oxyhemoglobin content is shown amounting
roughly to 1 per cent per day. The total hemoglobin content

Fig. 1.

of the original solution was 13.8 gm. per 100 cc. and the oxyhemo-
globin content 13.7 gm., while the latter had fallen to 11.05 gm.
at the end of 22 days.

BIBLIOGRAPHY.

1. Hüfner, G., *Arch. Physiol.*, 1901, suppl., 204, 205.
2. Mayet, *Compt. rend. Acad.*, 1889, cix, 156.
3. Bohr, C., *Skand. Arch. Physiol.*, 1892, iii, 50.
4. Torup. S., Die thermochemischen Reaktionen bei der Verbindung des
    Hämoglobins mit Sauerstoff und Kohlensäure, in Festschrift für
    Olof Hammarsten, Upsala, 1906.
5. Dudley, H. W., and Evans, C. L., *Biochem. J.*, 1921, xv, 487.
6. Adolph, E. F., and Ferry, R. M., *J. Biol. Chem.*, 1921, xlvii, 547.

7. Adair, G. S., Barcroft, J., and Bock, A. V., *J. Physiol.*, 1921, lv, 332.

8. Bohr, C., *Skand. Arch. Physiol.*, 1892, iii, 85.

9. Van Slyke, D. D., and Stadie, W. C., *J. Biol. Chem.*, 1921, xlix, 10.

10. Stadie, W. C., *J. Biol. Chem.*, 1920, xli, 237; Van Slyke, D. D., and Stadie, W. C., *J. Biol. Chem.*, 1921, xlix, 32.

11. Eggerth, A. H., *J. Biol. Chem.*, 1921, xlviii, 203.

GROWTH AND REPRODUCTION UPON SIMPLIFIED FOOD SUPPLY.

II. INFLUENCE OF FOOD UPON MOTHER AND YOUNG DURING THE LACTATION PERIOD.[*]

By H. C. SHERMAN and MARIE MUHLFELD.

(From the Department of Chemistry, Columbia University, New York.)

(Received for publication, May 17, 1922.)

In the preceding paper of this series[1] experiments were described in which it appeared that the quantitative proportion of milk in diets consisting of ground whole wheat and whole milk powder had a distinct influence upon the mother and young during the lactation period even in cases in which both diets would be accepted as adequate since both sufficed for growth, reproduction, and successful suckling of the young.

Thus two female rats, whose diets contained, respectively, one-sixth and one-third of milk solids, both raised good sized litters of young, but the larger proportion of milk in the diet resulted in more rapid growth of the young and less loss of weight on the part of the mother while suckling them.

Since, in our experience, individual variability constitutes an even more prominent factor in reproduction and lactation than in growth, we have extended the comparison of the efficiencies of these two diets for the support of lactation in the mother and growth in the suckling young to a large number of cases the average results of which are presented briefly in this paper.

For the convenience of the reader the diets used in the particular series of experiments with which this and the following paper[2]

___

[*] Published as Contribution No. 389 from the Department of Chemistry, Columbia University.

The expenses of this investigation were shared by the Department of Chemistry and the Borden Research Fund of Columbia University.

[1] Sherman, H. C., Rouse, M. E., Allen, B., and Woods, E., *J. Biol. Chem.*, 1921, xlvi, 503.

[2] Sherman, H. C., and Crocker, J., *J. Biol. Chem.*, 1922, liii, 49.

deal have been given alphabetical designations in order of in-
creasing proportions of milk in the food mixture.

Diet A (Laboratory No. 16) consists of one-sixth whole milk
powder and five-sixths ground whole wheat with sodium chloride
2 per cent of the weight of the wheat.

Diet B (Laboratory No. 13) consists of one-third whole milk
powder and two-thirds ground whole wheat with sodium chloride
2 per cent of the weight of the wheat.

Only distilled water was given and no bedding was used except
in the cases of females with young less than 2 weeks old, and then
only pure paper or cellulose. The rats had no access to wood
or any other material which could have been eaten in addition
to the regular diet. The diet was fed *ad libitum*, the ingredients
being so ground and mixed that the animals were obliged to
consume them in the exact proportions stated, and the amount of
food consumed was carefully determined and recorded in periods
of 1 week.

The general procedure was as follows. Rats breeding on experi-
mental diets are kept in galvanized wire cages in lots consisting
usually of one male and three to five females. Each rat is weighed
weekly at all times and more frequently as pregnancy advances.
Each female when apparently within a few days of giving birth
to young, is placed in a separate cage. As soon as the young are
born a new record of the mother's food consumption is begun and
the weighings of mother, young, and food are then made at
weekly intervals until the young are 4 weeks old when they are
separated from the mother and the latter is returned to her breed-
ing cage. Living in bare cages, our rats practically lose the nest-
making instinct, and even if offered bedding do not make nests in
advance of the birth of young. As a regular procedure we do
not offer bedding until after the birth of a litter is complete.
Some hours after the young have been born on the bare wire
cloth bottom of the cage, bedding is supplied in the form of
finely cut pure crêpe paper or pure cellulose in shredded form or
a mixture of these. The mother may or may not make a rough
temporary nest of this material. The cages are cleaned and fresh
bedding is supplied daily. This involves frequent but uniform
handling of the young after they are 24 to 36 hours old.

Doubtless the technique which we have adopted in order to insure cleanliness of cages, complete recovery of scattered food, and avoidance of any form of bedding which might be eaten by the rats, is somewhat severe and results in the rearing of a somewhat lower percentage of the young than might be reared by the same mothers on the same diets under the less rigorous conditions adopted in some other laboratories. Even if it be thought that our endeavor to insure a strictly quantitative record of food intake and the absence of roughage other than that supplied by the food, has resulted in unduly rigorous conditions and a consequently high rate of infant mortality, yet since these conditions are all uniform they cannot detract from the accuracy of our comparisons of different diets; and they do permit of a more strictly quantitative discussion of the efficiencies of different diets than would otherwise be possible.

TABLE I.

Comparison of Numbers of Young Born, and of Young Reared, by Ten Mothers on Each of Two Diets, A and B.

Diet.	Number of young born.		Number of young reared.		Percentage of young reared.
	Total.	Average.	Total.	Average.	
A	299	29 9	145	14 5	48
B	498	49 8	310	31 0	62

Influence of Diet upon Numbers of Young Born and Successfully Suckled.—The effects of the two diets here considered is best shown by a comparison of the complete reproduction records of the first ten females kept on each of the two diets, as shown in Table I.

Here the increased proportion of milk in the diet resulted in the birth of a larger number of young and also the rearing of a larger percentage of the young born.

A similar relationship was found when we compared the data of all the births during the year 1920; *viz.*, 167 litters born on Diet A and 402 litters born on Diet B. These latter figures are not here given in detail because their discussion is rendered somewhat cumbersome by the fact that both of these latter groups contain a large proportion of young mothers whose tendency to lose their first litters makes the percentage of infant mortality in

the averages for the year somewhat misleading unless explained in detail. It may, however, be emphasized that an experience with hundreds of litters born at all seasons of the year has fully confirmed the conclusions drawn from the data shown in Table I.

In this larger experience of 167 litters on Diet A and 402 litters on Diet B the increased proportion of milk in the diet of the mother evidently resulted both in the bearing and rearing of more young and in an increase in the number of young born per litter. On Diet A the most frequent number was six and the mean number was 5.51; on Diet B the most frequent number was seven and the mean was 6.47. As the difference between the means was over seven times its probable error, it is undoubtedly significant. A similar difference was found in the number of young raised per litter. As the number of litters raised by each mother was much larger among those receiving the higher proportion of milk in the diet, the net effect of this change in the quantitative proportions of foods in the dietary was the rearing of about double the number of young by each mother as illustrated by the typical data given in Table I.

Influence of Diet upon Maintenance of Mothers' Weight while Suckling Young.—As mentioned above, our technique involves weekly weighings of all rats in our colony. Hence by referring back we can always find the weight of each female at approximately the beginning of her pregnancy, 3 weeks before the birth of her young. With this weight we have compared the lowest weight observed during the lactation period as an indication of the efficiency of the diet in maintaining the mother while she is suckling her young.

When the number of young in the litter was small, and the demands of lactation relatively light, either of the diets here discussed was adequate for maintenance of the body weight of the mother; but with larger litters and consequently larger demands upon the suckling mothers, the diet containing the larger proportion of milk proved much more efficient in the maintenance of the body weight of the mother. Averaging for each diet all of the cases of mothers suckling litters of six young, the net difference in favor of Diet B was 12 gm. per capita or over 6 per cent of the mother's weight. For those suckling seven young the average difference was 35 gm. per capita or 18 per cent of the

mother's weight. For larger litters there are not sufficient numbers of cases of mothers of similar ages to permit of quantitative comparison.

It is plain that, when the demands of lactation are considerable, the quantitative proportions of milk in the two diets (each of which would ordinarily be adjudged adequate) becomes an important consideration in the maintenance of the mother as well as in the rearing of the young.

Influence of Diet of Mother upon Growth of Suckling Young.— In the experiments here described we did not weigh the young at birth but did weigh them at weekly intervals thereafter. From previous experience of our own and other laboratories we judge that healthy rats will vary but little from a range of 4 to 5 gm. at birth. At 4 weeks of age, however, our young rats of families on Diet A averaged 33.9 gm. (± 0.3 gm.), and those of families on Diet B averaged 42.3 gm. (±0.2 gm.). This increase of 8.4 gm. (±0.4 gm.), or 25 per cent in the average weight at weaning time, is far too large to be accidental and is plainly due to the increased percentage of milk in the food of the mother. In Table II the data are grouped according to the number of young in the litter. Since practically equal numbers of males and females were reared on each diet it is not necessary in this comparison to discuss the sexes separately.

It is evident from Table II that Diet A which appears fully adequate under ordinary tests fails to permit of a fully average growth of the suckling young especially in the larger families, whereas Diet B resulted in a fully average weight at weaning time even when the numbers of young were large.

Relation of the Proportion of Milk in the Diet to the Economy with Which the Food Is Used.—The foregoing data show that more and larger young were weaned with less drain upon the mother when the food of the family contained the higher percentage of milk. It would be of interest to know to what extent the better results are attributable to the consumption of more as well as better food, and to what extent to a more economical use of the food consumed. Too many factors enter into this problem to permit of our classifying all of our cases into comparisons in which only one factor shall vary at a time. This condition is, however, approximated in the case of the groups of

mothers raising litters of five, six, and seven young on the two
diets, respectively. These groups of females were of nearly
the same average initial weight and had nearly, if not quite,
completed their growth. From records of food consumption of
large numbers of normal rats in our colony it appears that young
adults require an average of 0.22 calories per gm. of body weight
per day for their own support. Making this allowance for the
maintenance of the mother we may calculate the rest of the food
actually consumed as chargeable to the rearing of the young.
(In the latter part of the 4 weeks period during which the young
remain with the mother, a part of the food furnished is, of course,

TABLE II.

Influence of Diet of Family on Size of Young at Weaning.

Number of young in litter.	Diet A.		Diet B.		Difference in favor of Diet B at 28 days.	
	Total number of young.	Average weight at 28 days.	Total number of young.	Average weight at 28 days.		
		gm.		*gm.*	*gm.*	*per cent*
2	4	38	4	43	+ 5	+13
3	12	36	15	43	+ 7	+19
4	36	38	32	40	+ 2	+ 5
5	65	34	80	41	+ 7	+21
6	66	35	132	41	+ 6	+17
7	35	34	154	44	+10	+29
8	16	26	88	43	+17	+65
9	0		45	41		
10	10	28	10	43	+15	+54

consumed directly by the young. For the comparisons which
we are here making we need not consider whether the food which
goes to nourish the mother and young is consumed in the first place
entirely by the mother or in part by her and in part by the
young directly.)

Taking then the records of food consumption of the three
most directly comparable groups of families on each of the two
diets, namely those having litters of five, six, and seven young,
deducting what the mothers would normally have eaten for their
own maintenance, and disregarding for the moment the changes
in body weight of the mother which actually occurred, it appears

that each gram of young rat reared to weaning time on Diet A cost 7.30 calories of extra food, while on Diet B this extra food cost was 6.74 calories. As these are averages for 27 families on Diet A and 60 families on Diet B and the difference appears consistently when the groups having five, six, and seven young are compared separately, the result cannot be accidental. It plainly establishes a more economical use of the diet containing the larger percentage of milk. The true difference in favor of this diet is seen to be considerably greater when account is taken of the fact that the body weight of the mother was at the same time much better supported by Diet B than by Diet A, the average difference in the cases here considered being 14.5 gm. in favor of Diet B for each nursing mother, or 7 to 8 per cent of her body weight. Hence it appears that the young rats of the families receiving the larger percentage of milk in their food were produced with greater economy both of the body material of the mother and of the calories of food consumed.

SUMMARY.

Breeding rats were fed upon diets containing respectively one-sixth whole milk powder to five-sixths ground whole wheat or one-third whole milk powder to two-thirds ground whole wheat. Young were successfully reared on both diets and both would be regarded as adequate for growth, reproduction, and successful suckling of the second generation. The larger proportion of milk in the second diet resulted in the following evidences of improved nutrition:

1. Increase in the number of young produced.

2. Increase in the percentage (and therefore also in the number) of young successfully suckled.

3. Better maintenance of the body weight by the mother while suckling the young.

4. Higher average weight of young at a standard weaning age of 4 weeks.

5. More economical utilization of the calories of food consumed (as well as of the body material of the mother) in the rearing of the young to weaning age.

GROWTH AND REPRODUCTION UPON SIMPLIFIED FOOD SUPPLY.

III. THE EFFICIENCY OF GROWTH AS INFLUENCED BY THE PROPORTION OF MILK IN THE DIET.*

By H. C. SHERMAN AND JOSEPHINE CROCKER.

(From the Department of Chemistry, Columbia University, New York.)

In the preceding papers of this series [1,2] it has been shown that food mixtures consisting of ground whole wheat and whole milk powder with distilled water and sodium chloride, are adequate for growth and reproduction in our experimental animals (rats) when one-sixth of the weight of the food mixture is milk powder, but that an increase in the proportion of milk to one-third of the solids of the food mixture results in more efficient nutrition as evidenced in better maintenance of the mother during the lactation period and the production and successful suckling of more and larger young.

The purpose of the present paper is to extend this study of the influence of the quantitative proportions of these simple mixtures by following their effects upon the rate and efficiency of growth of the young after weaning, and by considering also the effects of diets containing larger proportions of milk.

The series of diets of whole wheat and whole milk powder here referred to is as follows:

Diet A (Laboratory No. 16).—One-sixth whole milk powder and five-sixths ground whole wheat with sodium chloride 2 per cent of the weight of the wheat. Energy value 3.79 calories per gm.

Diet B (Laboratory No. 13).—One-third whole milk powder and

* Published as Contribution No. 390 from the Department of Chemistry, Columbia University.

[1] Sherman, H. C., Rouse, M. E., Allen, B., and Woods, E., *J. Biol. Chem.*, 1921, xlvi, 503.

[2] Sherman, H. C., and Muhlfeld, M., *J. Biol. Chem.*, 1922, liii, 41.

two-thirds ground whole wheat with sodium chloride 2 per cent
of the weight of the wheat.   Energy value 4.04 calories per gm.

Diet C (Laboratory No. 73).—Equal weights of whole milk
powder and ground whole wheat with sodium chloride 2 per cent
of the weight of the wheat.   Energy value 4.29 calories per gm.

Diet D (Laboratory No. 80).—Two-thirds whole milk powder and
one-third ground whole wheat with sodium chloride 2 per cent
of the weight of the wheat.   Energy value 4.55 calories per gm.

For reasons described in the preceding paper,[2] no bedding
was used and only distilled water was supplied.

The animals were allowed to eat *ad libitum* from weighed por-
tions of their respective food mixtures and the quantity of food
consumed was in each case determined by weighing the remaining
food at the same time with the weekly weighing of the rats.

The young rats were separated from their mothers and placed
upon the experimental diets at a standard "weaning" age of
4 weeks and thereafter weighed on a regular weekly weighing
day.   In the present discussion the weekly records are here
combined into a period of 4 weeks, the 5th to 8th weeks (inclusive)
of the life of the rat.   The average number of grams of body
weight gained per 1,000 calories of food consumed during this
definite 4 week period in the life of the rat is found to be a ser-
viceable method of comparing the efficiencies of the diets tested
in meeting one phase of nutritive requirement.

In presenting here the net results of this study we shall give
only the data which represent the averages of all comparable
records available at the time these calculations were made.   We
have, however, verified the conclusions thus reached by making
considerable numbers of experiments in which carefully matched
parallel lots of young animals were placed simultaneously upon
the different diets and kept side by side under exactly the same
conditions throughout.

The results of comparisons of general averages of all our data
and those obtained by comparing smaller numbers of more closely
matched animals agree.

Considering it best, therefore, to give the net results of all
our experience rather than take space for special comparisons
of matched individuals or lots, it becomes necessary to present
averages for the different diets in which the ratios of the sexes

among the experimental animals were not kept uniform. It is well known that the males grow somewhat faster than the females, but in our experience they eat correspondingly more so that the comparisons of the efficiencies of the different diets, which is the problem with which we are here concerned, may be made by observations either on males, females, or mixed lots of varying sex ratios. This is illustrated by the following comparison of two mixed lots of widely different sex ratios, both fed upon Diet B.

Lot 1247 consisting of one male and six females consumed 34 calories per rat per day and gained 71 gm. of body weight per 1,000 calories consumed; while Lot 1261 consisting of six males and one female consumed 40 calories per rat per day and gained 68 gm. of body weight per 1,000 calories consumed. The agreement between the figures representing the efficiency of the food

TABLE 1.

Efficiency of Growth on Different Diets.

Diet.	Number of lots.	Number of rats.	Average gain in gm. per 1,000 calories of food eaten.
A	32	163	54 ± 0.6
B	39	164	73 ± 0.8
C	34	164	74 ± 1.1
D	30	129	76 ± 1.1

as here considered (71 and 68, respectively) is as close as could be expected from this number of cases, if the sex ratios had been the same or if all the experimental animals had been of the same sex. Hence in Table I we present average data without distinction as to sex. Table I shows general average results for Diets A, B, C, and D.

It will be seen that Diet B showed marked superiority to Diet A as regards the efficiency with which the young rats grew upon these two diets, respectively. The difference per 1,000 calories consumed is here 19 gm. ($\pm$ 1.0 gm.), a difference which is certainly significant.

Whether efficiency of growth, as judged by this criterion, can be still further promoted by increasing the proportion of milk beyond that of Diet B cannot be stated with entire certainty. The average gain per 1,000 calories is higher for Diet C than for

Diet B, and higher for Diet D than for Diet C; but the differences in these latter cases are only about as large as their probable errors.

Apparently the diets in which milk furnished from one-third to two-thirds of the total solid matter of the food were for these conditions about equally efficient in supporting rapid growth, and certainly they were much more efficient than the diet composed of the same articles of food in which the proportion of milk was lower.

SUMMARY.

The efficiency of growth as influenced by diet is here studied by determining and comparing the gains in weight per 1,000 calories of food consumed during a fixed period of rapid growth in young rats.

Four diets, composed of whole milk powder and ground whole wheat in different proportions, were compared. Each diet was tested from 30 to 39 times and upon from 129 to 164 rats.

Diet A in which milk furnishes one-sixth of the solids or one-fifth of the calories of the food has been shown to be adequate; but growth is here shown to be much more efficient when the proportion of milk is increased so that, as in Diets B, C, and D, the milk furnishes from one-third to two-thirds of the solid matter, or 40 to 70 per cent of the total calories, of the food supply.

The method promises to be useful as a further means of studying and comparing the nutritive values of different adequate dietaries.

THE ESTIMATION OF LIPOID PHOSPHORIC ACID ("LECITHIN") IN BLOOD BY APPLICATION OF THE BELL AND DOISY METHOD FOR PHOSPHORUS.

By F. S. RANDLES and ARTHUR KNUDSON.

(From the Laboratory of Biochemistry, Union University Medical Department, Albany Medical College, Albany.)

Because of the comparative simplicity of the Bell and Doisy (1) method for the determination of phosphorus, it seemed desirable to apply this method to the determination of lipoid phosphoric acid ("lecithin") in blood. The nephelometric method of Bloor (2), while accurate and satisfactory in most respects has the disadvantage of requiring considerable time, as well as very careful technique. By adopting Bloor's (2) procedure for preparing and evaporating the alcohol-ether blood extract and the Bell and Doisy (1) method of digestion and color development, it appeared that a much simpler procedure would result.

A few trials of the new method on a composite sample of alcohol-ether extracts of blood sufficed to indicate not only the time-saving advantage of the procedure but, in addition, brought to light several sources of error in the colorimetric method which do not appear to have been previously observed in detail. The first difficulty appeared in trying to obtain check results on the same sample of blood extract. While the results agreed within 10 to 15 per cent, there seemed in some cases to be a poor match between the color of the unknown and that of the standard. This difficulty was thought to be connected with an excess acidity during the first stage of color development (as evidenced by excessive foaming on addition of the alkaline sulfite solution). Consequently, several experiments were made using standard solutions containing the same amount of phosphate solution but varying amounts of acid. As a result it was found that while the difference in acidity was not entirely responsible for the poor

Lipoid Phosphoric Acid in Blood

match it was in all probability the explanation of our inability
to obtain check results. Table I shows the effect of varying
acidity on the color intensity.

From Table I it is apparent that the color in Flasks 1 to
11 decreases with additional quantities of sulfuric acid. It is
of interest to note that within the range of acidity represented
in Flasks 5 to 8 the effect upon the color development is
not so decided as is the case outside of this range. This fact
serves to indicate the advisability of using 6 drops of acid
in the digestion rather than more or less and, furthermore,

TABLE I.

Effect of Varying Acidity on Depth of Color.

Flask No.	H_3PO_4 present.	Concentrated H_2SO_4.	Colorimeter reading.	Value found.	Error.
	mg.	drops	mm.	mg.	per cent
1	0.15	0	17.3	0.173	+15.3
2	0.15	1	18.2	0.165	+10.0
3	0.15	2	18.6	0.161	+ 7.3
4	0.15	3	19.4	0.154	+ 2.7
5	0.15	4	19.7	0.152	+ 1.3
6	0.15	5	19.9	0.151	+ 0.7
7	0.15	6	20.0	0.150	0.0
8	0.15	7	20.3	0.148	− 1.3
9	0.15	8	20.8	0.144	− 4.0
10	0.15	9	21.7	0.138	− 8.0
11	0.15	10	22.2	0.135	−10.0

Color developed in each case by the addition of 2 cc. each of molybdic
acid solution and hydroquinone solution, and after standing 5 minutes
followed by 10 cc. of carbonate sulfite solution. All were allowed to stand
5 minutes, made up to 25 cc. volume, and compared with No. 7.

shows the importance of carrying the digestion process always
to as near the same point as possible. By thus keeping the
number of drops of sulfuric acid in the tube after digestion within
4 to 6 drops and adding 6 drops to the standard the error due to
acidity falls within experimental limits.

Bell and Doisy (1) state that the color of the solution does not
fade at the same rate in the cups of the colorimeter as in the flask.
We studied, therefore, the effect of artificial light both during and
after color development, and at first were led to believe that
strong light intensified the color. If both cups of the colorim-

eter were filled at the same time with the same solution and set at the same height they were found to match. If, however, the cup on the right were removed, filled with a fresh solution from the flask, replaced in the colorimeter, and read immediately, the new sample was found to appear decidedly weaker in color. Furthermore, the new sample seemed to have a slightly different color, a greenish yellow tinge. But after standing for 5 minutes in the colorimeter the colors matched perfectly as regards both quality and intensity. That this result, however, was not due to the effect of light was later shown by repeating the experiment, and allowing the fresh solution to stand for 5 minutes in the dark before reading. It was found to match the other solution which had been exposed to the light. The fresh sample was then poured into a clean dry cup, poured back again, and read. The reading was again about 25 per cent high. The light was turned off for 5 minutes, readings were again made, and once more the colors matched. This was repeated a number of times, always with the same result; i.e., that pouring the solution from one container to another caused the color to appear weak in the colorimeter. This difference in color always disappeared, however, in 4 or 5 minutes. This interesting phenomenon appears to be due to the presence of bubbles in the solution which require about 5 minutes to "settle." It is especially noticeable when the solution has contained considerable acid before neutralizing and for that reason the bubbles are thought to be of carbon dioxide although no attempt has been made to establish definitely this point. The poor match encountered in studying the effect of acidity, and referred to above, was entirely due to the presence of these bubbles in the freshly poured-out solutions. To remedy the matter and avoid error, the standard should be poured back, and the cup refilled each time the solution in the "unknown" cup is changed; or if time permits 5 minutes may be allowed for the fresh solution to "settle" in which case it will not be necessary to disturb the standard.

Myers and Shevky (3) in their recent paper state that the Bell and Doisy method (1) will give accurate results when the standard solution does not contain more than 0.25 mg. of phosphorus per 100 cc. more than the unknown. This conclusion was reached from comparing solutions that had contained only the acid

necessary for the color reaction.   In using solutions that have an excess acidity during color development the range of accuracy seems to be still narrower.   It has been our experience that in cases where the reading of the unknown is more than 25 per cent above or below that of the standard a stronger or weaker standard should be used.   The required standard may be prepared after readings are started if necessary, as the color does not change for at least $\frac{1}{2}$ hour.

Method.

Reagents.—The reagents used are those described by Bell and Doisy (1).

Procedure.—5 cc. of whole blood or plasma are pipetted slowly into about 75 cc. of alcohol-ether mixture (consisting of 3 parts of alcohol and 1 part of ether, both redistilled) contained in a 100 cc. volumetric flask.   The flask should be shaken during the addition to avoid the formation of large clots of the precipitate. It is then immersed in boiling water and shaken well to avoid overheating.   As soon as the contents begin to boil the flask is removed, the mixture cooled to room temperature, made up to the mark with the alcohol-ether mixture, and filtered.   The filtrate may be preserved in a well stoppered bottle for a considerable period without deterioration.

For determination, 10 cc. of whole blood extract or 15 cc. of plasma extract are measured into a large Pyrex digestion tube (25×200 mm.), calibrated at 25 cc. and containing three glass beads.   The extract is then evaporated to dryness in a boiling water bath.   Unless the water bath is cold before immersing the tubes care must be taken to shake the tubes gently until boiling begins, to prevent the extract from boiling over.   To the dry residue in the tube 6 drops of concentrated sulfuric acid and 1 cc. of concentrated nitric acid are added.   Both acids must, of course, be free from phosphorus.   The mixture is then digested with a micro burner, at first over a low flame, then over a higher flame until the nitric acid is driven off and the remaining sulfuric acid is perfectly clear.   This digestion usually requires about 10 minutes.   After cooling for 1 or 2 minutes the sides of the tube are washed with about 5 cc. of distilled water and 2 cc. each of the molybdic acid solution and the hydroquinone solution

are added. After mixing and allowing to stand for 5 minutes 10 cc. of the alkaline sulfite solution are added and the whole is well mixed. After 5 minutes it is made up to the 25 cc. mark with distilled water and compared in the colorimeter with a standard made up as follows: 5 cc. of the standard monopotassium phosphate (containing 0.03 mg. of phosphoric acid per cc.) are added to a 25 cc. volumetric flask or tube graduated at 25 cc., containing 6 drops of concentrated sulfuric acid. The color is developed in the same manner as described for the unknown. In making readings with the colorimeter it is necessary to empty the standard cup into the flask and refill it each time the solution in the other cup is changed. Otherwise, the solution in the unknown cup must be allowed to stand for 5 minutes after being poured out before a reading is made.

If the standard is set at 20 mm. the calculation will be made according to the following formula:

$$S \times \frac{20}{R} \times \frac{20}{X} \times 100 = \text{mg. H}_3\text{PO}_4 \text{ per 100 cc. blood}$$

where S equals mg. of H_3PO_4 in amount of standard used, R equals reading of unknown, and X equals cc. of extract used.

Notes on the Method.—It was found that in order to get the best results the method of procedure should be standardized even to the smallest details. In the digestion, for example, it is advisable always to use the same number of glass beads and to carry the digestion to as near the same point as possible. For this purpose a tube of the same size as used in the digestion and containing the same number of beads and 6 drops of concentrated sulfuric acid may be set up beside the digestion tubes for comparison. This tube if calibrated at 25 cc. may be used for the development of the standard.

If a blood containing a normal amount of "lecithin" is being analyzed the standard may be made from 5 cc. of the standard phosphate solution described above. This should contain 0.15 mg. of phosphoric acid and would, therefore, cover the range 25 to 40 mg. of phosphoric acid per 100 cc. of blood. A weaker or stronger standard may be prepared very easily, however, if the unknown is found not to fall within the range of the prepared standard.

RESULTS AND DISCUSSION.

For the purpose of checking the accuracy of the method a
number of determinations were made using both the new colori-
metric method and the Bloor nephelometric method on the same
samples of human, dog, and rabbit blood. The results were
found to agree within 5 per cent as shown in Table II.

The advantages of this method as applied to the determination
of "lecithin" may be briefly summarized. The chief advantage
is the time saved. In making a large number of determinations
it is convenient to run about four digestions at a time. If this is

TABLE II.

Lipoid Phosphoric Acid per 100 Cc.

Specimen.	Colorimeter method.	Bloor method.
	mg.	*mg.*
Human blood...................................	39.25	40.5
" "	40.0	39.0
" " plasma.........................	25.75	26.37
Rabbit blood..................................	78.0	82.0
" " plasma.........................	55.6	55.0
Dog blood.....................................	61.6	64.8
" "	48.3	49.0
" " plasma.........................	54.0	53.5
" " "	37.7	35.5
Composite sample of human, dog, and rabbit blood and plasma...........................	49.3	49.06

done another series of four may be evaporating to dryness while
the determination is being carried out on the first four. In this
way determinations may be made at the rate of at least one every
10 minutes since 40 minutes is ample time for the digestion, color
development, and reading of a series of four samples. The di-
gestion time is cut to 10 minutes as compared with about 25 to
30 minutes in the Bloor method. The titration step in the latter
method is eliminated. Furthermore, the use of calibrated tubes
for the digestion makes it possible to carry out the whole operation
in the same tube, and save the time consumed in transferring
solutions from tubes to flasks with possible danger of loss and
contamination.

SUMMARY.

1. Varying the acidity during color development is found to have a marked effect.

2. The color is not affected by intense light but appears to be sensitive to mechanical agitation.

3. A procedure is described for applying the Bell and Doisy method to the estimation of lipoid phosphoric acid in blood.

BIBLIOGRAPHY.

1. Bell, R. D., and Doisy, E. A., *J. Biol. Chem.*, 1920, xliv, 55.
2. Bloor, W. R., *J. Biol. Chem.*, 1918, xxxvi, 33.
3. Myers, B. A., and Shevky, M. C., *J. Lab. and Clin. Med.*, 1921, vii, 176.

BUFFER SYSTEMS OF BLOOD SERUM.

By EDWARD A. DOISY, EMILY P. EATON, and K. S. CHOUKE.

(From the Laboratories of Biological Chemistry, Washington University School of Medicine, St. Louis.)

Loaned and Self-Possessed Serum Buffers.

In a previous paper (Doisy and Eaton, 1921), we have shown that the loss of hydrochloric acid from blood serum, by its migration into the cells, approximately accounts for the increase in the binding power of the serum for carbon dioxide when blood is exposed to increased tensions of that gas (Reaction 1). We considered that other acid radicals probably shift in a similar manner, but were unable to obtain any evidence of the passage of base across the cell membrane. Our work was incomplete in that we measured only the buffers loaned to the serum by the cells, and did not attempt to measure the "self-possessed" buffer value of the serum. Of the two types of reaction (Van Slyke, 1921),

(1) $BA + H_2CO_3 \rightarrow BHCO_3 + (HA) \rightarrow cells.$
(2) $B\ protein + H_2CO_3 \rightarrow BHCO_3 + H\ protein.$

our earlier experiments furnished data only for the first. In order to evaluate the relative importance of each of these buffer reactions, we have extended our work by a further study of the blood of four dogs and of two men.

As was pointed out in our previous paper, the corpuscles of blood occupy a larger volume as the tension of carbon dioxide in the gas with which they are in equilibrium is increased. Although this phenomenon was noted some time ago (von Limbeck, 1894–95), and its importance emphasized in our former paper,

it has not been considered in other recent investigations.[1] For
this reason it seemed probable to us that other estimates (Van
Slyke and Cullen, 1917; Fridericia, 1920) of the extent of the
migration of hydrochloric acid into the cells were too low.   The
use of anticoagulants is still another reason for thinking that the
values previously reported for chloride shift were too low.   Our
work has made it seem probable that all of the potential acids of
the serum take part in the migration, and that they enter into this
reaction to a degree which is dependent upon their concentration
and possibly upon their relative acid dissociation constants.
Samples of oxalated blood generally show that less than 50 per
cent of the increase of bicarbonate of serum (on treatment of
oxalated whole blood with increasing tensions of CO_2) is due to a
loss of chloride, whereas our results on defibrinated blood showed
the chloride shift to account much more closely for the gain in
bicarbonate.   It therefore seems fair to suppose that a shift of
oxalic acid explains the difference in behavior, and if this is true it
becomes important to avoid all such additions to blood in such
studies of acid-base equilibria.

In the experiments here reported we have attempted to analyze
the sources of alkali by which the blood combines with increasing
amounts of CO_2 and to estimate the quantitative contribution of
each.   Within a given range of pH, how much of the base for the
increase in serum bicarbonate is made available by the migration
of acids into the cells (reaction of Type 1, above)?   Is the migra-
tion of HCl alone sufficient to account for this quota, or do the
acids of other salts also migrate?   How much base is furnished
from the self-possessed, non-migrating buffers of the serum, and
to what relative extents do the serum proteins and the serum
phosphates participate?

The plan of our experiments was to determine the gain in serum
bicarbonate on equilibrating fully oxygenated, defibrinated blood

[1] L. J. Henderson has discussed this point rather briefly in some of his
recent papers (McLean, F. C., Murray, H. A., Jr., and Henderson, L. J.,
Proc. Soc. Exp. Biol. and Med., 1919-20, xvii, 181; Henderson L. J., *J.
Biol. Chem.*, 1920, xli, 427), but apparently has not attempted to make the
corrections for change in corpuscle volume as described in this paper.   The
mechanical models of Henderson and Spiro (Spiro, K., and Henderson,
L. J., *Biochem. Z.*, 1909, xv, 114) are very illuminating.

with 20, 40, and 60 mm. tension of CO_2. By determining also the gain in bicarbonate of separated sera similarly equilibrated with increasing tensions of CO_2, there is obtained the effect of the self-possessed serum buffers (Type 2). As indicated by Van Slyke in his treatment of the data on Joffe's blood, the difference between the two sets of values (for true serum and separated serum) represents the buffer action of Type 1, which is due to the presence of the corpuscles. The determination of chlorides in the true sera shows the amount of base liberated from NaCl by the migration of HCl. Since the earlier experiments gave no evidence of migration of base, by a comparison of the molar gain of bicarbonate due to "loaned buffer" with the molar loss of chloride in true sera, we attempt to determine the extent to which other acids migrate and thus contribute to the loaned buffer action. For one blood we determined also the hemoglobin and inorganic phosphates of the cells, which data allow the calculation of the relative amount of base furnished by each to the migrating acids.

The sera were also analyzed for inorganic phosphates and from this one may calculate the relative amount of base furnished by phosphate and protein buffers, the self-possessed buffers of the serum.

EXPERIMENTAL PROCEDURE.

The dog's blood for our experiments was drawn from the femoral artery while blood from the arm vein was used in the case of the two men. In each experiment it was defibrinated by stirring or shaking with beads. The fibrin was removed by filtering through gauze. Approximately one-half of the blood drawn was divided into three samples and equilibrated for 25 minutes at 38°C. with previously prepared gas mixtures containing about 20, 40, and 60 mm. of carbon dioxide. The other half was centrifuged to obtain "separated" serum, separate portions of which were similarly equilibrated with gas mixtures of the same composition as used with whole blood.

After equilibration of the whole blood, the samples were transferred to ordinary centrifuge and hematocrit tubes containing paraffin oil. The transfer was accomplished without contact with any other gas. On the completion of centrifugation the corpuscle volume was determined, and from the larger tubes serum was obtained for the chloride and carbon dioxide analyses. All

possible precautions were taken to avoid or minimize loss of CO_2 from samples of blood and serum from the completion of equilibration to the transfer to the apparatus for CO_2 determination. After equilibration of the "separated" serum, the samples were transferred under oil to tubes suitable for withdrawing 1 cc. portions for carbon dioxide analyses.

Carbon dioxide analyses were carried out by transferring 1 cc. of serum directly from the tubes to the cup of the Van Slyke macro apparatus (1917). The calculations were made according to the details suggested by Van Slyke and Stadie (1921). Chloride determinations were made on picric acid filtrates using the volumetric principles of the McLean-Van Slyke (1915) titration. In our hands the end-point appeared a bit sharper when the buffer and starch were used in separate solutions. In this case the starch solution was prepared fresh every few days. We also found that picric acid filtrates gave a sharper end-point under any conditions than the trichloroacetic acid filtrates used in our previous work.

After equilibration was completed, the gas mixtures in our saturators were analyzed with a Haldane burette. Phosphate analyses were made by the colorimetric method described by Bell and Doisy (1920).

Correction for Change in Corpuscle Volume.

The increase of corpuscular volume at the expense of water from the serum, which results from absorption of CO_2 causes an increase in the concentration of all substances in serum and the amount of this increase must be deducted before calculating loss of chloride and gain of bicarbonate caused by increasing CO_2 tension.

In order that we might plot our values of chloride and bicarbonate and thereby secure the advantages of the graphic method, we have modified the method of correction for change in the volume of the corpuscles. In our first paper, the correction was applied by calculating the concentration of chloride and bicarbonate that would exist due to the change in volume of corpuscles *if no migration of ions had occurred*. For example, in Experiment 1, we have the data given in Table I.

As corrected formerly, the values were obtained in the following way:

$$\frac{56.2}{55.6} \times 0.1122 = 0.1131 \text{ M NaCl} \qquad \frac{56.2}{55.6} \times 0.0164 = 0.0166 \text{ M NaHCO}_3$$

This method is undesirable in that it leaves different basic values with which to compare each value obtained at higher tensions of CO_2, and such results for three tensions cannot be plotted on the same curve. It is preferable to correct all observed concentrations to *one basic volume* taking for this purpose the volume of the serum at the lowest carbon dioxide tension. The values for bicarbonate and chloride at the higher CO_2 tensions are merely multiplied by the necessary factor to give the respective concentrations that would exist in the serum had its volume not changed due to the taking up of water by the corpuscles.

TABLE I.

Experiment 1.

CO_2, mm	17 6	39 3	61 6
Serum, per cent of total blood volume	56 2	55 6	55 0
$NaHCO_3$, molar concentration	0 0164	0 0219	0 0257
NaCl, molar concentration	0.1122	0 1097	0 1085

TABLE II.

Correction for Change of Corpuscle Volume.

	Former method.		New method.	
CO_2, mm	17 6 to 39 3	17 6 to 61 6	17 6 to 39 3	17 6 to 61 6
NaCl-loss, *mols*	0.0037	0 0062	0 0036	0 0060
$NaHCO_3$-gain, *mols*	0 0053	0 0090	0 0053	0 0088
Percentage to loss of chloride	70	69	68	68

From the data just given, the correction by this method is:

$$\frac{55.6}{56.2} \times 0.1097 = 0.1086 \text{ м NaCl} \qquad \frac{55.6}{56.2} \times 0.0219 = 0.0217 \text{ м NaHCO}_3$$

The values for the highest tension of CO_2 are calculated in the same way using in this case the factor $\frac{55.0}{56.2}$ and the concentrations found by analysis. As shown by comparison in Table II, the numerical values obtained by the two methods are substantially the same. The new procedure has the advantage that the results may be plotted on a single graph.

RESULTS.

The experimental data from the different samples of blood are given in detail in the protocols. The values for serum bicarbonate and chloride are corrected for change in serum volume as above described, and are brought together in Table III. In order to

TABLE III

*Loaned Buffer; Loss of Chloride and Gain of Bicarbonate.**

	CO_2	pH	$NaHCO_3$	Difference in $NaHCO_3$.	NaCl	Difference in NaCl.
	mm.		mols	mols	mols	mols
Dog 1.	17.6	7.57	0.0164		0.1122	
	39.3	7.34	0.0217	0.0053	0.1086	0.0036
	61.6	7.22	0.0252	0.0088	0.1062	0.0060
Dog 2.	18.5	7.51	0.0152		0.1122	
	42.5	7.30	0.0212	0.0060	0.1079	0.0043
	66.0	7.18	0.0244	0.0092	0.1042	0.0080
Dog 3.	18.5	7.53	0.0159		0.1110	
	37.3	7.34	0.0204	0.0045	0.1076	0.0034
	74.8	7.12	0.0243	0.0084	0.1042	0.0068
Dog 4.	20.0	7.50	0.0159		0.1182	
	44.3	7.27	0.0207	0.0048	0.1142	0.0040
	76.0	7.11	0.0237	0.0078	0.1091	0.0091
Man 1.	23.3	7.47	0.0175		0.1110	
	43.0	7.33	0.0230	0.0055	0.1071	0.0039
	72.5	7.17	0.0267	0.0092	0.1043	0.0067
Man 2.	16.7	7.65	0.0189		0.1103	
	35.2	7.44	0.0241	0.0052	0.1065	0.0038
	63.8	7.27	0.0290	0.0101	0.1020	0.0083

* Bicarbonate and chloride values have been corrected for change in corpuscular volume.

compare the relative participation of the various buffer factors, it is necessary to choose an arbitrary pH range, and to calculate the effect of each factor within this range. Without change in hydrogen ion concentration, the effect of the self-possessed serum buffers is, of course, zero, the buffer action being limited to that depending upon migration of acid to and from the cells. We have chosen the range of pH 7.45 to 7.25 as the basis of our calculations,

and in doing so it may be noted that we undoubtedly exaggerate the relative participation of the self-possessed buffers, which as pointed out by Van Slyke (1921) play less and less of a rôle under physiological conditions the smaller the difference in pH between arterial and venous blood. This range was chosen to encompass the varying values which appear to occur naturally, if not physiologically, and especially in order to minimize the errors in the determinations.

Since some of our experimental data commonly fall without this pH range, it is necessary to plot them in the form of curves and
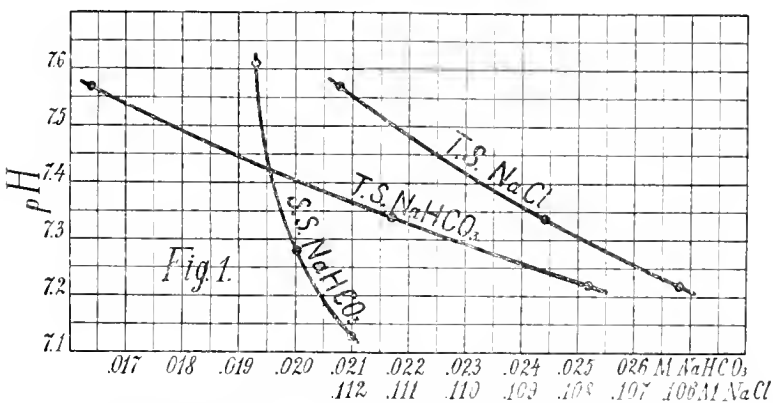

FIG. 1. Data are taken from Experiment 1 which was conducted on dog's blood. Chloride and bicarbonate values of true sera corrected for the change in volume of the corpuscles are plotted against pH.

obtain the desired values by interpolation. The most convenient method of treatment is to plot the molar concentrations of bicarbonate and of chloride of the true sera (corrected for change in serum volume) and the molar concentrations of bicarbonate of the separated serum against the pH which is calculated from the ratio of free and combined CO_2. The corrected data, given in Table III are plotted[2] in this way in Figs. 1, 2, and 3. From the

[2] Only three of the six charts are presented. The concavity to the abscissæ is greatest in Fig. 2, while the convexity is greatest in Fig. 3. All the curves of the other experiments occupy intermediate positions. It should be noted that if the true serum bicarbonate curve is convex to the abscissæ, the chloride curve varies from a straight line in the same direction. Generally this has been true in all our experiments. At present, we do not understand the significance of these variations.

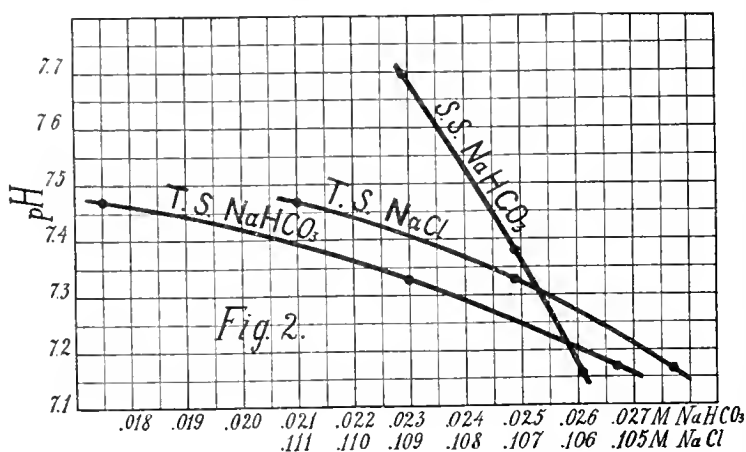Fig. 2. Data are taken from Experiment 5 which was conducted on human blood. Chloride and bicarbonate values of true sera corrected for the change in volume of the corpuscles are plotted against pH.
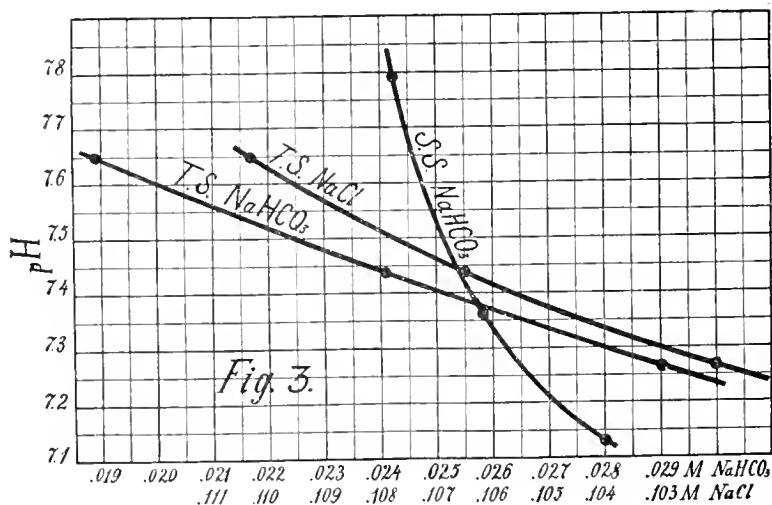

Fig. 3. Data are taken from Experiment 6 which was conducted on reduced human blood. Chloride and bicarbonate values of true sera corrected for the change in volume of the corpuscles are plotted against pH.

Doisy, Eaton, and Chouke 69

TABLE IV.

Serum Buffers, pH 7.45 to 7.25.

pH	True serum. Molar NaHCO₃.			Separated serum. Molar NaHCO₃.			True serum. Molar NaCl.			Percentage of base supplied from:		
	7.45	7.25	Gain.	7.45	7.25	Gain.	7.45	7.25	Loss.	Self-possessed buffers	HCl migration.	Unaccounted for
Dog 1...	0.0188	0.0242	0.0054	0.0195	0.0201	0.0006	0.1105	0.1069	0.0036	11	67	22
" 2...	0.0169	0.0225	0.0056	0.0163	0.0168	0.0005	0.1112	0.1066	0.0046	9	82	9
" 3...	0.0181	0.0222	0.0041	0.0154	0.0161	0.0007	0.1095	0.1061	0.0034	17	83	0
" 4...	0.0169	0.0211	0.0012	0.0225	0.0233	0.0008	0.1175	0.1138	0.0037	19	88	−7
Man 1...	0.0187	0.0250	0.0063	0.0245	0.0257	0.0012	0.1103	0.1056	0.0046	19	75	6
" 2...	0.0238	0.0296	0.0058	0.0253	0.0266	0.0013	0.1067	0.1016	0.0051	23	88	−11
Mean...										16	80.5	3+

curves in these figures, we read off the changes in concentration corresponding to the change in pH from 7.45 to 7.25 and these values are given in Table IV. Taking the data from Experiment 1 as an example, it will be seen (Table III) that on passing from a pH of 7.45 to 7.25, the true serum gained 0.0054 mols of bicarbonate, of which only 0.0006 mols or 11 per cent is due to the buffers of the separated serum (the self-possessed buffers), the remaining 0.0048 mols or 89 per cent being due to the presence of corpuscles (the loaned buffers). Of the latter, the disappearance of NaCl (by migration of HCl into the cells), 0.0036 mols, accounts for three-fourths, or for 67 per cent of the total gain. The remaining 22 per cent of the total gain (0.0012 mols) which is not accounted for in this experiment is perhaps due to the migration of other acids from serum salts into the cells. All of the other experiments show somewhat smaller fractions to be unaccounted for (from $+ 0.0005$ to -0.0006 molar). The difficulty in the accurate measurement of small differences between much larger quantities is such that the variations between extremes is no greater than might be expected. The effect of small errors in individual values is considerable, as may be illustrated by the following calculations on the serum of Dog 1, Experiment 1.

Assuming that the basic value 0.1122 M NaCl is correct, let us see what effect an error of -0.0005 M or 0.5 per cent produces in our estimation of the loaned buffer value. The corrected value at 39.3 mm. of CO_2 $(0.1097 - 0.0005 = 0.1092$. $0.1092 \times \dfrac{55.6}{56.2}$ $= 0.1080)$ becomes 0.1080 instead of 0.1086 and the loss of chloride is now 0.0042 instead of 0.0036. This accounts for $\left(\dfrac{0.0042 \times 100}{0.0053} = \right)$ 79 per cent of the total gain of bicarbonate instead of the 68 per cent actually found. Viewed in another way, an error of 0.5 per cent in the determination of one value produces a difference of about 12 per cent in the result. It is thus apparent that too much must not be expected from individual determinations. We, therefore, deem it more desirable to consider only the mean values. According to the average of all of our data, the buffer value of serum in the pH range of 7.45 to 7.25 is loaned to it to the extent of 80+ per cent by a migration of hydrochloric acid into the corpuscles; while the effect of the non-migrating self-

possessed buffers amounts to 16 per cent. This leaves 3 per cent unaccounted for and this is probably due to a migration of acids other than hydrochloric. The evidence presented by de Boer (1917) on the migration of SO_4^{--}, our results concerning phosphates, the behavior of oxalated blood already referred to, and particularly the fact that a large migration of H_2CO_3 into the corpuscles takes place, would seem to indicate that such a conclusion is justified.

We can feel fairly certain that we know the nature of all of the buffer reactions of the serum when the state of oxidation of the hemoglobin remains unchanged. In a recent paper, L. J. Henderson and coworkers (1919-20) state that when the oxygenated

TABLE V.

Alkali Furnished by Phosphate Buffer System.

Experiment.	Total gain of NaHCO₃ in separated sera. pH 7.45 to 7.25.	Total inorganic P of sera.	Base yielded by Na₂HPO₄ pH 7.45 to 7.25.	Percentage of total alkali supplied by Na₂HPO₄.	
				Separated sera.	True sera.
	molar	*molar*	*molar*	*per cent*	*per cent*
Dog 1.	0 0006	0.00129	0.0001	17	1 9
" 2.	0 0005	0.00174	0 00014	28	2 5
" 3.	0 0007	0.00084	0 00007	10	1 7
" 4.	0.0008	0 00119	0.0001	12	2.4
Man 1.	0 0012	0.00087	0 00007	6	1 1
" 2.	0 0013	0.0010	0.00008	6	1 4
Mean...........................				13	1.8

blood is reduced isohydrically about 60 per cent of the increased capacity of serum to bind CO_2, is due to a migration of chloride. Although this might seem to indicate some other loaned buffer mechanism, we shall attempt to show in a later paper that at least 90 per cent of the increased capacity is actually due to a shift of HCl. The lower estimate is due to neglecting the change in concentrations produced by the swelling of the corpuscles.

By means of our inorganic phosphate determinations we may calculate the participation of the serum phosphate in the self-possessed buffer action. By using the Hasselbalch equation,

$$pH = pK_1 + \log \frac{[Na_2HPO_4]}{[NaH_2PO_4]}$$

we can solve for the ratio of disodium to dihydrogen phosphate at pH 7.45 and 7.25 and from our data on serum inorganic phosphate calculate how much of the separated serum buffer value is due to the phosphate system. The fraction which this represents of the total base supplied by all buffers of separated serum shows the relative participation of the phosphates.

The data on this point are given in Table V. According to these calculations from 6 to 28 per cent of the self-possessed buffer action may be due to the phosphates; the remainder being due to salts of proteins and amino- and other organic acids. The serum phosphates appear to supply only from 1 to 3 per cent of the base contributed by the *total* serum buffers.

SUMMARY.

A series of experiments on samples of blood of four dogs and two men show that between the pH range af 7.45 and 7.25, the base furnished for the increase of bicarbonate in serum comes from the sources indicated (mean results):

(*a*) "Self-possessed", non-migrating serum buffers: 16 per cent. Of this, phosphates supply 1 to 3 per cent.

(*b*) "Loaned" buffer, due to presence of corpuscles: 84 per cent. Of this, migration of HCl into corpuscles liberates 80 per cent. The remaining 3 per cent is probably liberated from the salts of other acids, by migration of the latter into the corpuscles.

Protocols.

The blood was defibrinated by one of the usual procedures. Each gas-equilibrating mixture consisted of air plus CO_2 (except Experiment 6). With the exception of Experiment 4, no hemolysis occurred, and in this case it was very faint.

Experiment 1—Dog 1.

	True serum.			Separated serum.		
CO_2, *mm*	17 6	39.3	61 6	18.8	41.8	62.4
Total CO_2, *vols. per cent*	37 9	51.9	62.0	44.5	47.9	51.6
Dissolved CO_2, *vols. per cent*..	1.25	2.80	4 38	1.34	2.98	4.44
$BHCO_3$, *vols. per cent*	36 6	49 1	57 6	43.2	44.9	47.2
pH.............................	7.57	7 34	7.22	7.61	7.28	7.13
NaCl, *mols*	0 1122	0 1097	0.1085			
Serum, *per cent*...............	56 2	55 6	55 0			
Inorganic P, *mg*.............				4.0		

Experiment 2—Dog 2.

	True serum.			Separated serum.		
CO_2, *mm*	18.5	42.5	66.0	19.7	39.8	65.0
Total CO_2, *vols. per cent*	35.4	51.4	61.1	37.7	40.8	44.8
Dissolved CO_2, *vols. per cent*	1.32	3.03	4.70	1.40	2.83	4.63
$BHCO_3$, *vols. per cent*	34.1	48.4	56.4	36.3	38.0	40.2
pH	7.51	7.30	7.18	7.51	7.23	7.04
NaCl. *mols*	0.1122	0.1099	0.1078			
Serum, *per cent*	48.2	47.3	46.6			
Inorganic P, *mg*				5.4		

Experiment 3—Dog 3.

	True serum.			Separated serum.		
CO_2, *mm*	18.5	37.3	74.8	18.0	41.2	71.6
Total CO_2, *vols. per cent*	37.0	48.8	61.3	35.0	39.5	43.1
Dissolved CO_2, *vols. per cent*	1.32	2.66	5.32	1.28	2.93	5.10
$BHCO_3$, *vols. per cent*	35.7	46.1	56.0	33.7	36.6	38.0
pH	7.53	7.34	7.12	7.52	7.20	6.97
NaCl, *mols*	0.1110	0.1088	0.1073			
Serum, *per cent*	56.2	55.6	54.6			
Inorganic P, *mg*				2.6		

Experiment 4—Dog 4.

	True serum.			Separated serum.		
CO_2, *mm*	20.0	44.3	76.0	16.1	41.6	67.6
Total CO_2, *vols. per cent*	37.1	50.3	61.0	50.5	54.2	58.6
Dissolved CO_2, *vols. per cent*	1.42	3.15	5.41	1.15	2.96	4.81
$BHCO_3$, *vols. per cent*	35.7	47.1	55.6	49.3	51.2	53.8
pH	7.50	7.27	7.11	7.73	7.34	7.15
NaCl, *mols*	0.1182	0.1161	0.1140			
Serum, *per cent*	56.7	55.8	54.3*			
Inorganic P, *mg*				3.7		

* Probably erroneous; change larger than expected.

Experiment 5—Man 1.

	True serum.			Separated serum.		
CO_2, mm...................	23.3	43.0	72.5	18.1	41.2	71.2
Total CO_2, *vols. per cent*......	40.9	55.4	66.4	52.6	58.7	63.5
Dissolved CO_2, *vols. per cent*..	1.66	3.06	5.16	1.29	2.93	5.07
$BHCO_3$, *vols. per cent*........	39.2	52.3	61.2	51.3	55.8	58.4
pH..........................	7.47	7.33	7.17	7.70	7.38	7.16
NaCl, *mols*..................	0.1110	0.1087	0.1065			
Serum, *per cent*..............	54.1	53.3	53.0			
Inorganic P, *mg*.............				2.7		

Experiment 6—Man 2.

	True serum.*			Separated serum		
CO_2, mm..................	16.7	35.2	63.8	15.6	44.7	82.0
Total CO_2, *vols. per cent*......	43.5	57.1	71.3	55.3	61.0	68.6
Dissolved CO_2, *vols. per cent*..	1.19	2.50	4.54	1.11	3.18	5.83
$BHCO_3$, *vols. per cent*........	42.3	54.6	66.8	54.2	57.8	62.8
pH..........................	7.65	7.44	7.27	7.79	7.36	7.13
NaCl, *mols*..................	0.1103	0.1076	0.1047			
Serum, *per cent*..............	56.5	55.9	55.0			
Inorganic P, *mg*.............				3.1		

* The true serum of this blood was obtained by equilibration with H_2 $+ CO_2$. Approximately 95 per cent of the hemoglobin was in the reduced form.

BIBLIOGRAPHY.

Bell, R. D., and Doisy, E. A., *J. Biol. Chem.*, 1920, xliv, 55.
de Boer, S., *J. Physiol.*, 1917, li, 211.
Doisy, E. A., and Eaton, E. P., *J. Biol. Chem.*, 1921, xlvii, 377.
Fridericia, L. S., *J. Biol. Chem.*, 1920, xlii, 245.
von Limbeck, R., *Arch. exp. Path. u. Pharmacol.*, 1894-95, xxxv, 309.
McLean, F. C., and Van Slyke, D. D., *J. Biol. Chem.*, 1915, xxi, 361.
McLean, F. C., Murray, H. A., Jr., and Henderson, L. J., *Proc. Soc. Exp. Biol. and Med.*, 1919-20, xvii, 180.
Van Slyke, D. D., *J. Biol. Chem.*, 1917, xxx, 347.
Van Slyke, D. D., *Physiol. Rev.*, 1921, i, 141.
Van Slyke, D. D., and Cullen, G. E., *J. Biol. Chem.*, 1917, xxx, 289.
Van Slyke, D. D., and Stadie, W. C., *J. Biol. Chem.*, 1921, xlix, 1.

STUDIES ON ENZYME ACTION.

XX. THE PROTEASE ACTIONS OF MALIGNANT HUMAN AND RAT TUMOR EXTRACTS AT DIFFERENT HYDROGEN ION CONCENTRATIONS AND IN THE PRESENCE OF VARIOUS SALTS.

By K. GEORGE FALK, HELEN MILLER NOYES, AND
KANEMATSU SUGIURA.

(*From the Harriman Research Laboratory, the Roosevelt Hospital, and the Huntington Fund for Cancer Research, Memorial Hospital, New York.*)

INTRODUCTION.

The chemical changes which occur in living matter are profoundly influenced, and even may be said to be controlled, by enzymes and their actions.[1] Thus, different enzymes in different parts of a living organism may produce different products from one and the same mixture supplied to the different parts. Strictly speaking, this statement should be reversed. Since different products are obtained in different parts of a living animal, for example, from the circulating blood, the presence of various enzymes in these different parts is inferred, since enzymes, at present, are known only by their actions. Without entering farther into these questions in the present connection, the importance of enzyme actions in the chemical changes of living matter is definitely recognized. As a logical development, a modification of such enzyme actions might well be expected in pathological conditions. This investigation was therefore begun to study certain enzymes which may be involved in malignant growths. The results in this paper are limited to the study of the proteolytic enzyme, and refer to the actions obtained with various human malignant tumors as well as the Flexner-Jobling rat carcinoma. The effect on the protease actions of different hydrogen ion con-

---

[1] *Cf.* for example Falk, K. G., Catalytic action, Chapter VII, A chemical interpretation of life processes, New York, 1922.

centrations, the determination of the optimum hydrogen ion concentration, the change in action with time and with concentration of substrate and of enzyme material, and the effect of a number of neutral salts on the action at the optimum hydrogen ion concentration, were studied.

Experimental Methods.

Preparation of Materials for Enzyme Tests.—The solutions and mixtures used for the enzyme experiments were obtained from human tumors and rat tumors. With the former, the material was prepared from specimens removed in surgical operations, within 2 to 3 hours after the operations. With the latter, the neoplasm was removed from the animal after killing it with ether according to the method described in detail by Sugiura and Benedict.[2] In both cases, after the removal of all non-neoplastic material, including fibrous tissue, fat, muscle, etc., the tumor was cut into small pieces, washed with physiological salt solution, and dried by means of filter paper. A small portion of the tumor was dried in an air oven at 102° in order to determine the water content. The remainder was weighed, macerated with sea sand, treated with a definite quantity of water or saline solution, shaken thoroughly, and after the addition of a small quantity of toluene, placed in an ice box over night. The mixture was then centrifuged and filtered, either through paper or with suction through a Gooch crucible containing a thin mat of long fibred asbestos.

The tumors of human origin were obtained at more or less irregular intervals as the surgical material presented itself through the courtesy of Dr. E. T. Leddy of the Staff of Memorial Hospital. The characters of the growths as given in Table I were obtained from the records of the Pathological Department of the Hospital. The rat tumors were obtained from albino rats, both male and female, which were inoculated with the Flexner-Jobling rat carcinoma in the usual way and allowed to grow for a period of from 3 to 6 weeks.[2] The term $\frac{FRC}{86A}$ for example, indicates that the tumor used was derived from the 86th generation, Series A.   The rats were fed on wheat bread soaked in whole milk, fresh cabbage or carrots, and fresh tap water *ad libitum.*

[2] Sugiura, K., and Benedict, S. R., *J. Cancer Research*, 1920, v, 373.

Protease Methods.—The proteolytic actions of the tumor extracts were studied by the formol and the Van Slyke amino nitrogen methods on casein and on a peptone preparation.[3] Not all extracts were tested by both methods with both substrates, but enough experiments were carried out to make definite conclusions possible.

For the Van Slyke amino nitrogen method the micro apparatus was used and the customary precautions taken.[4] The use of casein as substrate caused a certain amount of trouble. In solutions more acid than pH 7.0, the presence of casein in suspension necessitated some care in order to run the requisite quantity of mixture into the reaction bulb, while the introduction of the casein into the acid mixture in the reaction bulb in every case resulted in the casein flocculating out. In order to have comparable conditions and also to cause the reaction to proceed to completion as far as practicable, the reaction bulb was shaken for 8 minutes in every determination. Either 2 or 3 cc. were used in the determinations.

In carrying out the determinations by the Sörensen formol method, 15 to 25 cc. of the mixtures and 0.1 N sodium hydroxide solution were used. Phenolphthalein was used as indicator. The titrations were divided into two steps, first to a definite pink color (direct), then after the addition of 10 to 15 cc. of 35 per cent formaldehyde solution neutralized toward phenolphthalein, to a definite pink color again (formol). The results presented in this paper are comparative; that is to say, the enzyme actions were obtained by subtracting from the results with enzyme-substrate mixtures, the sums of the separate results of the enzyme-water (or solution) and substrate-water (or solution).

Preparation of Solutions.—The pH values of the various solutions were determined by means of indicators and the standard solutions recommended by Clark.[5] The latter were checked up potentiometrically. A number of the peptone solutions were tested potentiometrically and as a result of these tests the indicators were chosen which gave the most satisfactory color comparisons. The substrate solutions in the earlier experiments were

[3] Obtained from Fairchild Bros. and Foster, New York.

[4] Van Slyke, D. D., *J. Biol. Chem.*, 1913-14, xvi, 121.

[5] Clark, W. M., The determination of hydrogen ions, Baltimore, 1920.

prepared by direct weighings of the different peptone and casein samples. Later, a stock solution was prepared for any one series and hydrogen ion concentration, and suitable portions were pipetted from this.

Casein was used as substrate as a type of complex protein, while the peptone was used because of its more ready handling under the given conditions. The results and conclusions with these two substrates were essentially the same.

In all the experiments except those involving neutral salt actions, 0.1 N sodium hydroxide or hydrochloric acid was used to bring the solutions for the enzyme tests to the required hydrogen ion concentrations. In the neutral salt tests, the hydroxyl or hydrogen compound of the metallic or acid component of the neutral salt was used in order to avoid the introduction of a different salt into the mixture.

Toluene was present in every mixture throughout the tests.

EXPERIMENTAL RESULTS.

Protease Actions.

General Data.—The data relative to the natures, amounts, and treatments of the tumors for the purpose of obtaining extracts for the protease tests are given in Table I.

The amounts to which the various extracts, dialyzed and not dialyzed, were diluted in the different protease tests are not given directly in the table. The final results of such dilutions are shown, however, in the last column, in which are given per cc. of the mixtures tested the quantities of original tumors extracted.

The extracts in the first eleven experiments, in Experiments 30, 31, and 32, and in part of Experiment 28 were used directly. The other extracts were dialyzed over night in collodion bags against running tap water. The pH of this tap water was 7.0 to 7.2, and its temperature varied between 10 and 20° at the different times. The volumes of the solutions increased between 20 and 50 per cent in the dialyses. These increases were taken into account in calculating the final concentrations.

The filtered rat tumor extracts were colorless to very light yellow. Some of the human tumor extracts were red in color

(because of blood), but on dilution in preparing the mixtures for the tests the color was found not to interfere. The pH values of the various extracts varied between 6.6 and 7.0. With some of the extracts the addition of acid to bring them to the required pH value produced a faint turbidity or cloudiness.

The special treatments and explanations with reference to the protease actions will be given in connection with the separate results. Unless stated differently, the reactions were allowed to proceed for 22 hours at 38° and the actions then determined.

The presentation of the results of the protease actions presents certain difficulties. The concentration of the substrate can be kept uniform in the various series of experiments, but every tumor extract is likely to be different even if the concentrations of the solid tumors as extracted had been kept contant. Each series with any one tumor preparation should, strictly speaking, therefore, be considered separately. The presentation of the individual experiments is, however, impracticable, in view of their large number. The results will therefore be given in the first instance in terms of a common unit of volume and concentration of substrate, that is, as amounts of protease action per cc. of mixture tested; and in the second instance as relative actions by considering the maximum action observed in any one series as 100 per cent and calculating the other actions of this series in terms of this. In the second way, the differences in the enzyme preparations are eliminated, and the relative actions studied on a common basis.

The Van Slyke method gives directly the increase in the concentration of amino groups formed on proteolysis, after proper corrections for blanks have been made. Nothing is indicated as to other reactions which may have occurred.

The formol method gives the concentration of acid produced in the reaction. This acid may be formed as a result of the hydrolysis of a peptide linking, and also of any other decomposition or hydrolysis not necessarily accompanied by the simultaneous formation of an amino group as in the peptide hydrolysis. Also, the formol titration may indicate the production of ammonia, present as an ammonium salt before the addition of formaldehyde, the acid component of the ammonium salt being determined in the formol titration. The change in hydrogen ion concentration of the mixture would show any pronounced acid or alkali formation

TABLE I.

Data Relative to the Treatment of Tumors for Protease Experiments.

Experiment No.	Source of tumor.	Character of tumor.	Fresh tumor. gm.	Water content of tumor. per cent	Extracting solution. Nature.	Extracting solution. Amount. cc.	Filtered tumor extract treatment.	Tumor content of solution in protease experiments. mg. per cc.
2	Human.	Epidermoid carcinoma of cervix.	2.0	82.2	0.9 per cent NaCl.	50		2.1
3a	Rat.	$\frac{FRC}{87A}$, active tissue.	2.66	85.2	0.9 "	50		3.1
3b	"	$\frac{FRC}{87A}$, necrotic tissue.	2.29	84.6	0.9 "	50		2.3
5a	Human.	Neurogenic sarcoma of thigh.	21.0	82.4	0.9 "	100		19.1
5b	"	Neurogenic sarcoma of thigh.	21.0	82.4	Water.	100		19.3
6	"	Papilloma of bladder.	5.1	81.6	"	100		4.1
7	"	Squamous carcinoma of antrum.	2.23	81.3	"	50		1.8
8	Rat.	$\frac{FRC}{87A}$	11.3	81.2	"	100	a. Unfiltered. b. Filtered through paper. c. Filtered through asbestos.	3.0

9	Rat.	$\frac{FRC}{87A}$	10.2	83.5	Water.	100		2.7
10	"	$\frac{FRC}{87A}$ and $\frac{FRC}{87B}$	30.1	83.0	"	100		24.1
11	Human.	Neurosarcoma of back.		87.5	"	100		31.5
12	Rat.	$\frac{FRC}{89B}$	45.1	81.9	"	150		69.4
13	Human.	Papillary adenocarcinoma of parotid gland.	68.3	78.3	"	100	Dialyzed.	98.8
14	Rat.	$\frac{FRC}{89B}$	26.9	85.1	"	100	"	29.8
15	"	$\frac{FRC}{89B}$	15.7	83.5	"	100	"	46.5
16	"	Spontaneous tumor; spindle-celled sarcoma.	12.3	86.0	"	100	"	37.5
17	Human.	Neurosarcoma of arm.	13.3	86.5	"	100	"	17.2
18	Rat.	$\frac{FRC}{90A}$	22.6	83.8	"	100	"	58.6

TABLE I—*Concluded.*

Experiment No.	Source of tumor.	Character of tumor.	Fresh tumor.	Water content of tumor.	Extracting solution.		Filtered tumor extract treatment	Tumor content of solution in protease experiments.
					Nature.	Amount.		
			gm	*per cent*		*cc.*		*mg. per cc.*
19	Human.	Endothelioma of sacrum.	93.4	81.9	Water.	250	Dialyzed.	42.5, 85 0 170 0, 102
20	Rat.	$\frac{FRC}{90C}$	28.4	85.6	"	100	"	32.1 to 128.4
21	Human.	Myxosarcoma of leg.	39.8	87.2	"	100	"	129.0
22	"	Glionyoma of uterus.	150 0	79.0	"	300	"	156.3 and 195.4
23	Rat.	$\frac{FRC}{91A}$	41.5	83.6	"	150	"	114 6
24	"	Normal muscle tissue of hind legs.	56.5	78 0	"	200	"	121 3
25	"	Normal muscle tissue of hind legs.	61 9	75 9	"	200	"	126.9
26	"	$\frac{FRC}{91C}$	51.0	82.9	"	150	"	110.7
27	"	$\frac{FRC}{96A}$	30.0	84.2	"	100	"	83.7

28	Rat.	FRC/96B	30.3	85.0	Water.	100	{a. Dialyzed. b. Not dialyzed.	61.2 61.2
29	"	FRC/96B	12.9	83.7	"	50	Dialyzed.	60.4
30	Human.	Melanoma of femoral triangle.	118.0	79.6	"	300	Dialyzed; not dialyzed.	98.7
31	Rat.	FRC/96C	29.0	85.5	"	100		55.1
32	Human.	Papillary adenocarcinoma of cervix.	31.5	82.6	"	100		69.0
34	Rat.	FRC/98B	33.8	81.1	"	150	Dialyzed.	60.7
35	"	FRC/98B	36.9	84.7	"	150	"	80.8
36	"	FRC/99A	25.2	84.0	"	100	"	61.9
37	"	Normal muscle tissue of hind leg.	51.4	84.7	"	150	"	111.6
39	Human.	Papillary adenocarcinoma of ovary.	83.7	83.9	"	200	"	137.4

in the reaction. The formol method evidently, therefore, does not yield results theoretically as simple and as clear-cut as the Van Slyke method.

In the experiments in which the mixtures at the beginning of the actions were treated with acid or alkali so that their pH values varied from 4.0 to 9.0, the direct titrations brought all the mixtures to the same pH (very nearly 8.5), and the formol titrations then gave the carboxyl groups produced in the proteolytic actions. These formol titrations would be expected to give smaller values than would be obtained in the corresponding Van Slyke amino nitrogen determinations for the hydrolysis of the peptide linkings corresponding to the proteolyses. Each series would be complete in itself, but a more or less constant factor would represent the differences between the two. If the formol titration is started at pH 7.0, however, the Van Slyke and formol methods give essentially the same results as shown in the study of the hydrolysis of gelatin by Northrop,[6] and in the results which will be communicated in this paper.

In order to have as great a uniformity as possible, the results will be given in terms of milligrams of nitrogen in the form of amino groups produced by the protease actions in 100 cc. of the mixtures at the temperature 38° and in the time indicated, corrected for enzyme and substrate blanks. They are obtained directly from the determinations by the Van Slyke method.   In the formol method, the hydrolysis of the peptide linking is assumed to be the only reaction occurring.   In the series in which the actions at different hydrogen ion concentrations were studied, the results for the direct titrations will be indicated as milliequivalents of acid (or alkali for the negative acid values) per 100 cc. of mixture, and for the formol titrations as milligrams of nitrogen per 100 cc.   Both titrations were carried out with phenolphthalein as indicator.   The formol titration results are not the same numerically as the Van Slyke results, but the comparative changes and the optima observed are the same.   In the other series, the formol method was carried out with the solutions initially at pH 7.0.   Although the titrations were carried out in two steps, direct and formol, with phenolphthalein as indicator in each, the results will be combined and presented as one titration

[6] Northrop, J. H., *J. Gen. Physiol.*, 1920–21, iii, 715.

in terms of milligrams of amino nitrogen formed per 100 cc. of mixture.

Protease Actions at Different Hydrogen Ion Concentrations.—The first problem studied included the determination of protease activities at different hydrogen ion concentrations with a view to finding the optimum conditions for the actions. Preparations from Tumors 2 to 11 were used for this purpose. None of these extracts was dialyzed before testing. Table II shows some of the experimental results which were obtained. In all these experiments 1 cc. of the mixture contained 8 mg. of peptone or casein. In Table III and Fig. 1 are presented the results for all the experiments in which more or less complete series were obtained at a number of different hydrogen ion concentrations. A number of series were obtained with results for pH 4.0, 6.0, and 8.0, only. These confirm in general the more complete series and will not be given in detail.

The main conclusion to be drawn from these results is that a definite optimum for the actions is observable at pH 7.0, with a more gradual decrease toward pH 8.0 than toward pH 6.0.

A few explanations and further conclusions with reference to these experiments may be given. Extracts of Tumor 5 were prepared both with water and with physiological salt solution. These were tested at pH 4.0, 6.0, and 8.0, and gave practically identical results. All the subsequent extractions were therefore carried out with water only. Extracts of Tumors 2 and 3 were also tested only at pH 4.0, 6.0, and 8.0, and are not included in the results. The negative values of a number of the direct titrations, especially with peptone, are of interest. The hydrolysis evidently produced an excess of the alkaline products (probably ammonia) over the acid. The amounts in most cases, especially with the initial pH values of 5.0, 6.0, and 7.0, are extremely small, but unquestionably are correct. They do not, however, appear to effect the formol titrations. Experiment 7 gave unaccountably large negative direct titration values.

The extracts of Tumors 8 and 9 were tested for protease actions unfiltered, filtered through paper, and filtered through asbestos. No regular difference was observed to indicate that either method of filtration removed active material.

TABLE II

Protease Actions of Tumor Extracts at Different Hydrogen Ion Concentrations.

Experiment No.	Substrate	Method	pH 4.0	5.0	6.0	7.0	8.0	9.0
6	Peptone.	Direct, milli-equivalents acid per 100 cc...		0	−0.01	−0.10		−0.11
		Formol, mg. N per 100 cc...		1.3	3.7	4.1	4.1	0.9
7	"	Direct, milli-equivalents acid per 100 cc...		−0.16	−0.29	−0.18	−0.14	−0.34
		Formol, mg. N per 100 cc...		2.2	3.9	5.0	4.9	2.7
8a	"	Direct, milli-equivalents acid per 100 cc...		−0.01	−0.08	−0.11	−0.31	−0.16
		Formol, mg. N per 100 cc...		3.4	3.1	4.3	2.7	2.4
8b	"	Direct, milli-equivalents acid per 100 cc...		0.06	−0.02	0.04	0.15	−0.11
		Formol, mg. N per 100 cc...		2.0	4.1	5.3	3.9	1.5
8c	"	Direct, milli-equivalents acid per 100 cc...		0.02	−0.01	−0.06	−0.14	−0.31
		Formol, mg. N per 100 cc...		1.6	3.8	5.2		2.2
8a	Caseín.	Direct, milli-equivalents acid per 100 cc...		0.01	0.12	−0.02	−0.06	0.01
		Formol, mg. N per 100 cc...		1.4	1.5	3.4	0.8	0.8
8b	"	Direct, milli-equivalents acid per 100 cc...		0.01	0.05	−0.01	−0.13	−0.12
		Formol, mg. N per 100 cc...		0.6	0.9	1.5	1.2	0.8
8c	"	Direct, milli-equivalents acid per 100 cc...		0.08	0.07	−0.03	−0.15	−0.09
		Formol, mg. N per 100 cc...		1.6	0.9	1.6	1.2	0.5
10	Peptone.	Van Slyke, mg. N per 100 cc...	1.8	4.4	8.0	12.2	11.2	6.4

raction

Comparison between the experimentally observed values in the different experiments is not feasible because of the different concentrations of tumor materials in the various mixtures. However, of those quoted so far, all except Experiment 10 were of the same order of magnitude as far as concentration of extracted material was concerned, and gave actions also of the same order. Experiment 10 with tumor in greater concentration gave considerably greater actions.

The variation in protease action with the hydrogen ion concentration is brought out clearly in Table III (and Fig. 1) which also permits of a comparison of the different experiments from this point of view. The maximum action in any one series is placed at 100 and the actions at the other hydrogen ion concentrations calculated in terms of this. Although there are several minor discrepancies, the general relation of a distinct optimum at pH 7.0 is brought out clearly.

Rate of Protease Actions.—A number of series of experiments were carried out to determine the amounts of the actions after definite time intervals. Extracts from tumors, or Preparations 19, 22, 23, 24, and 25 were used. 1 cc. of mixture contained 20 mg. of peptone in every case, except Experiment 19 where 16 mg. were present per cc. Both the formol and Van Slyke methods were used. The tests were run at pH 7.0. The formol results include the sums of the direct (which were very small in every case) and formol titrations. In experiments of this nature occasional irregularities are apparently unavoidable.

The experimental results expressed as milligrams of nitrogen formed by the protease actions per 100 cc. of mixtures, corrected for blanks, are given in Table IV. These results were plotted, and since the determinations by the formol and Van Slyke methods in those cases where both were used gave practically the same results, such series were combined. Fig. 2 shows two of the curves obtained; those for Extracts 22 and 24. Table V then gives the values for the actions at more regular time intervals as taken from the various curves for the experimental results shown in Table IV.

Although it is difficult to compare enzyme actions from different materials in a quantitative manner, some conclusions may be

drawn relative to the kinetics of the reactions. The data presented in Table V will be used.

Extracts 19 and 22 were obtained from tumors of human origin, Extract 23 from a tumor of rat origin, Extracts 24 and 25 from normal rat tissue. Very nearly the same weights of tumor were extracted per cc. of final mixture.

Comparing the actions of the last three extracts (material from rats), it is seen that the tumor protease showed much greater activity than the protease of normal tissue. No special significance should be attached to this fact, however, since the cell

TABLE IV.

Experimental Results on the Rates of Protease Actions of Extracts.

Extract 19.		Extract 22.			Extract 23			Extract 24.			Extract 25		
Time.	Van Slyke method	Time.	Van Slyke method	Formol method	Time.	Van Slyke method	Formol method	Time.	Van Slyke method	Formol method	Time.	Van Slyke method	Formol method.
min.		*min.*			*min.*			*min.*			*min*		
0	0	0	0	0	0	0	0	0	0	0	0	0	0
55	1.2	115	9.2	9 1	190	11 2	8 3	210	2 1	3 4	215	0 4	5 4
105	1 6	185	10 5	10 7	290	11.2	11 0	285	2 9	2 9	1,710	22 4	19 0
250	5.4	300	13.9	17 9	1,335	26 9	26 4	1,675	11 5	13 1	1,730	21 1	
370	5.4	1,295	25 4	23 8	1,435	26 5	28 0	2,895	19 9	16 6	2,790	20 9	22.7
1,675	14.6	1,395	22 7	23 0	1,735	29 0	29 1	3,135		17 4			
1,735	15.2	1,775		24 9	3,070	39 0	39 0						
3,135	19.1	2,845	29 1	29.3									

characters were entirely different. Except for Extract 25, none of the actions appeared to have reached a limit.

Attempts to apply methematical equations to the kinetic relationships of these protease actions are as inconclusive as with most enzyme actions. This may be illustrated by the application of Schütz's rule[7] to the results given in Table V. Schütz's rule states that the amounts of enzyme action are proportional to the square roots of the times of actions. Table VI gives the values for K as derived from the expression
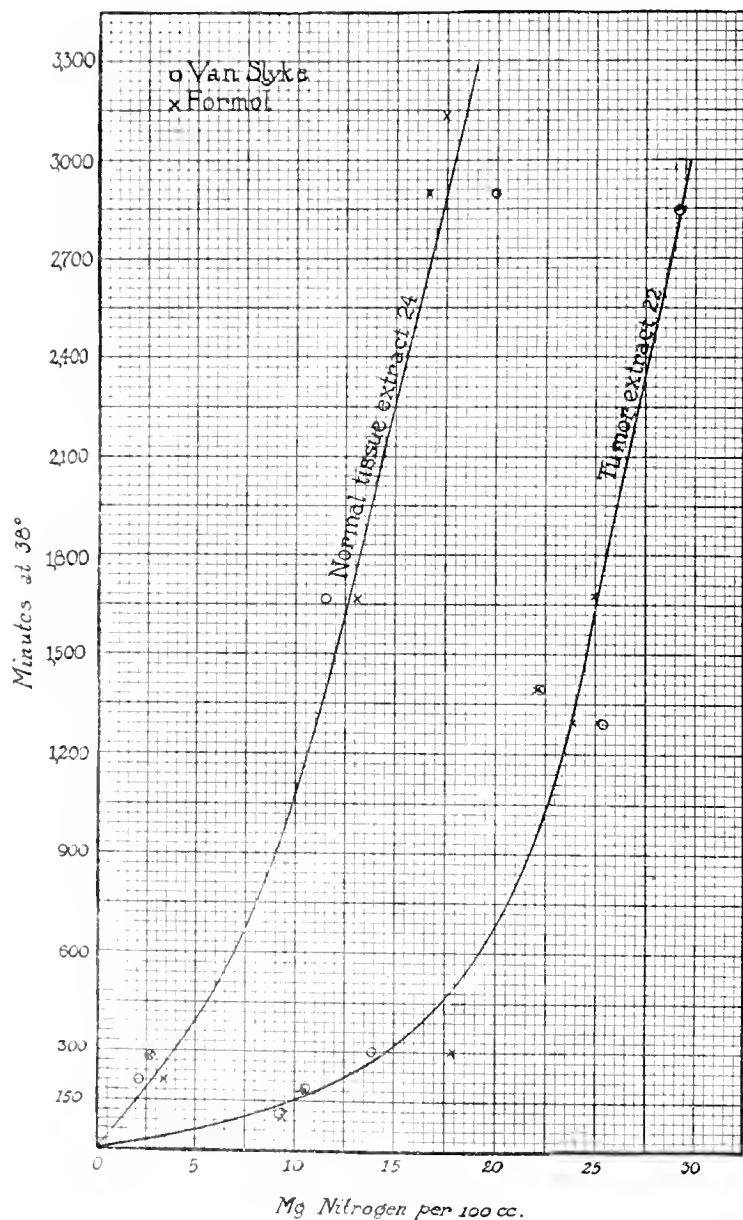
[7] Schütz, E., *Z. physiol. Chem.*, 1885, ix, 577.

FIG. 2. Rates of protease actions of extracts on peptone at pH 7.0. Times of actions (minutes) plotted as ordinates; amounts of actions (as milligrams of amino nitrogen produced per 100 cc. of mixtures), as abscissæ.

Curve 22 represents results obtained with tumor extract 22; Curve 24, results with normal tissue extract 24.

$$K = \frac{x}{\sqrt{E}\ T}$$

in which x represents the change in time, T, for enzyme concentration, E. E is taken to be constant in each series.

TABLE V.

Rates of Protease Actions of Extracts Taken from Curves of Experimental Results.

Time.	Human.		Rat tumor.	Normal rat.	
	Extract 19.	Extract 22.	Extract 23	Extract 24	Extract 25.
min.					
0	0	0	0	0	0
60	1.0	5.2	3.0	0.9	1.6
120	1.9	8.5	5.5	1.7	3.1
240	3.7	12.8	9.6	3.2	5.9
480	6.7	17.7	15.2	5.8	10.3
720	9.2	20.3	19.5	7.8	13.6
960	11.2	22.0	22.7	9.3	16.2
1,200	12.7	23.2	25.5	10.6	18.1
1,440	14.0	24.2	27.7	11.7	19.5
1,920	15.8	26.0	31.6	13.7	21.3
2,400	17.3	27.6	34.8	15.6	22.0
2,880	18.5	29.3	37.9	17.5	22.1

TABLE VI

Values of the Constant of Schütz's Rule Derived from the Results of Table V.

Time.	Extract 19.	Extract 22	Extract 23	Extract 24	Extract 25.
min.					
60	0.13	0.67	0.39	0.12	0.21
120	0.17	0.78	0.50	0.16	0.28
240	0.24	0.83	0.62	0.21	0.38
480	0.31	0.81	0.69	0.27	0.47
720	0.34	0.76	0.73	0.29	0.51
960	0.36	0.71	0.73	0.30	0.52
1,200	0.36	0.66	0.72	0.30	0.51
1,440	0.37	0.64	0.73	0.31	0.51
1,920	0.36	0.59	0.72	0.31	0.49
2,400	0.35	0.56	0.71	0.32	0.45
2,880	0.35	0.55	0.71	0.33	0.41

It was shown by Northrop[8] in interpreting the mechanism of Schütz's rule, that the rule involves the assumptions that the concentrations of the products of reaction are large compared to the concentration of enzyme, and that the quantity of substrate remains fairly constant. These conditions hold only after the reaction has proceeded to a certain extent, and during the time that a certain amount of action occurs. In the reaction between pepsin and albumin, Northrop found that the constant calculated according to Schütz's rule first increased with the time, then remained constant for various lengths of time, and then decreased. Exactly the same behavior is shown by the results given in Table VI for the extracts from the three sources. All the actions showed initial increases, then constancy for different periods, and then more or less irregular decreases. Northrop developed an equation to include such factors as varying enzyme concentration, which would be expected to hold for practically the whole course of the reaction. The application of this equation, involves, however, the knowledge of the final value of the enzyme action (hydrolysis in this case). The results obtained in this investigation do not permit of the determination of these final values, since there are, at least in four of the five series, no indications that the reactions had approached completion. It might be thought that the consideration of this expression as one containing two unknowns, the constant and the final value of the actions, evaluating perhaps by the method of least squares, would give results of interest. This method of treatment, while employed at times, really assumes the fact which is being tested.

The results given in this section may perhaps be interpreted more satisfactorily when the mechanism of enzyme actions shall have been formulated more completely. Until then, the results must be considered as empirical contributions, which agree with the observations of others as far as comparison at the present time is possible.

Relations between the Protease Actions and the Changes in Enzyme and Substrate Concentrations.—Two series of experiments were carried out with extracts of Tumors 19 and 20 (one of human and one of rat origin) in which the concentrations of enzyme material and of substrate were varied. The relative enzyme concentra-

[8] Northrop, J. H., *J. Gen. Physiol.*, 1919–20, ii, 471.

tions were in the ratios 1:2:4 in both series. With Tumor Extract 19, the peptone concentrations were in the ratios 1:2.5:5, ranging from 2.65 to 13.2 mg. of peptone per cc. of final mixture; with Tumor Extract 20, their concentrations were in the ratios 1:2.5:5:10; ranging from 2.0 to 20 mg. per cc. of final mixture. The experiments were carried out for 22 hours at 38°. The Van Slyke method was used for the determinations.

Table VII shows the results of the protease actions expressed as milligrams of amino nitrogen produced per 100 cc. of mixture.

TABLE VII.

Effect of Change in Concentration of Enzyme and Substrate on Protease Actions of Tumor Extracts.

	Concentration of peptone.			
	1 0	2 5	5 0	10 0
Concentration of tumor extract 19.				
1	4.4	6.6	7.7	
2	5.3	9.8	16.4	
4	9.3	14.6	24.2	
Concentration of tumor extract 20.				
1	3 9	5.8	11.1	22.3
2	4.8	11.1	16.5	25.1
4	5.7	14 8	20.8	39.5

In order to study the effects of changes in concentrations of enzyme and of substrate satisfactorily, it is advisable to follow the time necessary to produce the same percentage change in substrate in the different cases. This was not done in the present instance, but, even so, some conclusions may be drawn from the results. Table VIII shows the values of K calculated by means of Schütz's rule from the equation

$$K = \frac{x}{\sqrt{E} \cdot A}$$

in which x represents the extent of the action, and E and A the relative concentrations of enzyme and substrate, respectively.

The average value of the constant of Schütz's rule from the results in Table VIII for Tumor Extract 19 is 4.4, for Tumor Extract 20 it is 4.5. There is quite a variation in the individual

values, and some of them are obviously incorrect. It is not easy
to draw any general conclusions as to possible trend of the values
with the limited number of results at hand, but perhaps it may
be said that the values in any one series tend to increase with
increased concentration of substrate. At any rate, in view of the
possible errors of the experiments and the use of amounts of action
in equal times instead of the more accurate use of lengths of time
for equal percentage actions, Schütz's rule indicates a rough
approximation to a probable explanation of the mechanism of the
actions. This is especially true when taken in connection with
the results of the time action determinations given in the pre-
ceding section, and points to the fact that some more general

TABLE VIII

Values of the Constant of Schütz's Rule Derived from the Results of Table VII.

	Concentration of peptone.			
	1 0	2 5	5 0	10.0
Concentration of tumor extract 19.				
1	4 4	4 2	3.4	
2	3.7	4.4	5.2	
4	4.7	4 6	5 4	
Concentration of tumor extract 20.				
1	3 9	3.7	5.0	3.5
2	3.4	5.0	5.2	5.6
4	2.9	4.7	4.6	6.2

theoretical explanation of the mechanism of these enzyme actions
such as that outlined by Northrop is correct and that Schütz's
rule is a special and limited case of the general formulation.

*Influence of a Number of Neutral Salts on the Protease Actions
of Tumor Extracts.*—The protease actions of a number of tumor
extracts were tested after the addition of various neutral salts.
In place of giving the detailed experimental results for the indi-
vidual tests, the actions will all be given in relative terms, that is,
the percentage actions in the presence of the different concentra-
tions of the different salts with the action in the absence of the
salt in the given series as unity. In every series, an experiment
with the extract without added salt was run. The values of the
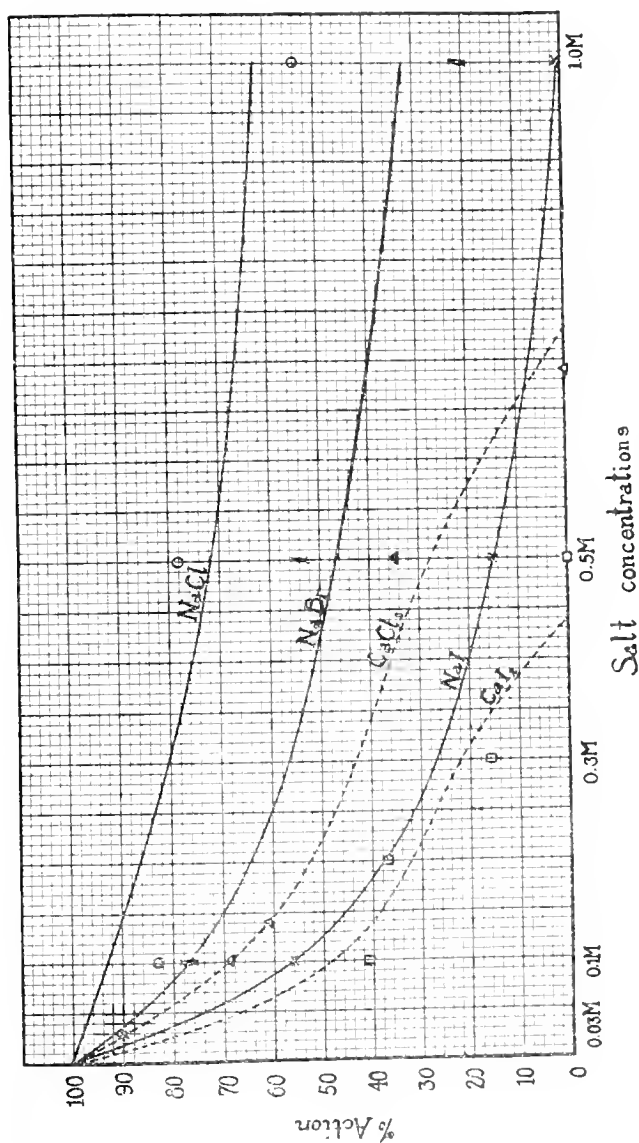protease actions for these direct tests were, as a rule, of the same

Fig. 3. Protease actions at pH 7.0 in the presence of salts. Concentrations of salts plotted as abscissa; actions expressed as percentages of actions in the absence of salts, as ordinates.

general order of magnitude as the results already given.   The relative actions in the presence of the salts can therefore be approximated.

TABLE IX.

Relative Protease Actions of Tumor Extracts in the Presence of Salts.

Salt.	Tumor extract No	Method	Molar concentrations of added salts.								
			0	0.03	0.10	0.14	0.20	0.30	0.50	0.69	1.0
NaCl	14	Van Slyke.	100		67				66		24
	18	" "	100		84				84		
	22	" "	100		85				85		53
	26	" "	100		84				72		69
	26	Formol.	100		94				84		70
NaF	14	Van Slyke.	100		76				55		48
	13	" "	100		95				95		42
NaBr	15	" "	100		62				52		0
	16	" "	100		92				48		0
	17	" "	100		76				62		64
NaI	15	" "	100		58				0		0
	16	" "	100		50				0		0
	17	" "	100		59				46		3
KCl	26	" "	100		91				73		65
	26	Formol.	100		94				84		61
LiCl	18	Van Slyke.	100		100				93		
CaCl$_2$	27	" "	100		58	50			26	0	
	19	" "	100	91					64		
	29	" "	100		58						
	27	Formol.	100			72			14	0	0
	29	"	100			75					
	30	Van Slyke.	100	79	55						
	30	Formol.	100	100	85						
	31	Van Slyke.	100		71						
	31	Formol.	100		82						
	32	"	100		69						
CaI$_2$	19	Van Slyke.	100		41		37	16	0		

The experiments were run in every case for 22 hours at 38°. Each final mixture contained the tumor in the concentration in-

dicated in Table I and 8 mg. of peptone per cc. Unless stated to
the contrary, every tumor extract was dialyzed against tap water
for 18 hours, and the mixtures were made up with these dialyzed
solutions to which the salt, etc., were added. The final mixtures
were made up to have a pH of 7.0.

The averages of the separate results for the different salts are
given in Table X.

TABLE X

*Average Values for the Relative Protease Actions of Tumor Extracts in the
Presence of Salts.*

Salt	Molar concentrations of salts.							
	0	0 03	0 10	0 14	0 20	0 30	0 50	1 0
NaCl	100		83				78	54
NaF	100		86				75	45
NaBr	100		77				54	21
NaI	100		56				15	1
KCl	100		93				79	63
LiCl	100		100				93	
CaCl$_2$	100	90	69	61			35	0
CaI$_2$	100		41		37	16	0	

In order to facilitate comparison between the different actions,
the results for sodium chloride, sodium bromide, sodium iodide,
calcium chloride, and calcium iodide were plotted and are shown
in Fig. 3.

A study of the results given in Table IX shows considerable
variations of the various tumor extracts under conditions of added
salt where similar results, as expressed in terms of percentage
actions, might be expected. In part, these variations are undoubt-
edly due to experimental errors, since the quantities measured are
comparatively small. At the same time, variations between the
influences exerted by different added substances on these protease
actions would naturally be expected with tumors derived from
different individuals, rat as well as human. Especially with human
tumors of different types, as shown in the descriptions in Table I,
would differences in actions be expected to occur. The data at
hand are not extended enough to permit of a possible classification

of the actions according to the type and nature of tumor, so that for the purposes in view an average of the results for any one salt has been taken. Under the circumstances, the general agreement of the relations between the effects of the added salts on the protease actions of the extracts of tumors from such very different sources is striking.

The average values for the actions of the various salts which are given in Table X clearly indicate the following facts:

1. The protease action was decreased in every case with increasing concentration of salt.

2. Sodium chloride and sodium fluoride exerted comparatively small retarding effects, the actions being decreased only one-fourth at 0.5 molar concentration, and one-half at molar concentration.

3. Potassium chloride and lithium chloride exerted smaller inhibiting actions, if anything, than did the sodium chloride and sodium fluoride, although the smaller number of results with the former do not make the results with them quite as satisfactory.

4. Sodium bromide retarded the actions to a greater extent than did sodium chloride, about one-fourth at 0.1 molar, one-half at 0.5 molar, and four-fifths at molar concentration.

5. Sodium iodide showed still greater retarding effects, the actions decreasing to about one-half at 0.1 molar concentration.

6. Calcium chloride retarded the actions to a much greater extent than the sodium chloride, but not as much as the sodium iodide for the corresponding molar concentrations.

7. Calcium iodide retarded the actions to the greatest extent of the salts which were studied. This was to be expected in view of the retarding actions of sodium iodide and calcium chloride.

In view of the retarding actions of the calcium salts, the protease actions of some of the tumor extracts were determined after various treatments with calcium salts. A definite time factor was observed. The experiments in which the tumor extracts contained calcium chloride for some time before their activities were tested, showed markedly less protease actions on the peptone.

A study of the growth of transplanted Flexner-Jobling rat carcinoma after treatment with various solutions, will be spoken of in the following section. In view of the relations found in this study, the protease actions of tumor extracts in the presence of salts mixed in the proportions used in the Locke-Ringer solution

were determined. Table XI gives the results of these determinations with the salts present in the concentrations given in such a solution, in three times these concentrations, and also in a solution containing only the sodium chloride. The results are presented as percentage actions, with no added salt present taken to be 100.

TABLE XI.

Relative Protease Actions of Tumor Extracts in the Presence of Salt Mixtures.

Experiment No.	Method.	No salt	0.15 M NaCl	0.45 M NaCl.	0.15 M NaCl 0.003 M CaCl₂ 0.003 M KCl	0.45 M NaCl 0.009 M CaCl₂ 0.009 M KCl
34	Van Slyke.	100		55	69	64
	Formol.	100	86	64	100	78
35	Van Slyke.	100	72	82	94	
	Formol.	100	99	83	100	85
36	Van Slyke.	100	97	76	89	75
	Formol.	100	86	69	91	72
39	Van Slyke.	100	73	82	89	74
	Formol.	100		86		93
Averages...............		100	86	75	90	77

Calcium chloride, in the concentrations 0.003 and 0.009 M, did not retard the actions. In fact, a slight increase was noticed as compared with the actions in the absence of the salt, but this may have been due to experimental inaccuracies. The results shown in Table XI indicate clearly that the addition of calcium and potassium chlorides to the sodium chloride solution in the relative proportions of the Locke-Ringer solution, did not reduce the retarding action of the sodium chloride on the protease actions. The concentrations of the calcium and potassium chloride were too small to cause appreciable retarding actions in themselves. A comparison of these results with the results of Tables IX and X and with the corresponding curves in Fig. 3 brings this out clearly. In other words, there is no antagonistic salt action observable with the protease of tumor extracts.

DISCUSSION OF RESULTS.

The discussion of the experimental results involves: first, a comparison of the protease actions of the tumor extracts with the actions of proteases from different sources; and second, a comparison of the tumor protease actions with transplantation phenomena of the Flexner-Jobling rat carcinoma.

The protease actions found do not differ in any significant way from the protease actions observed by other workers with material from similar sources.[9] It must be remembered that the present investigation did not include directly the study of the chemical composition of tumors.

An important point which should be emphasized is that as far as these studies went, the protease actions of human and rat tumor extracts were essentially the same. This applies especially to the optimum hydrogen ion concentration for the actions and to the behavior of neutral salts, the inhibiting effect of calcium in both series being striking. The tumor tissue protease was found to be somewhat more active than the normal tissue protease. This agrees with the observation of Abderhalden,[10] who also found that extracts of normal and of tumor tissue of rats and mice hydrolyzed polypeptides and silk peptone in different ways. However, it may be recalled that enzymes of normal tissue from different parts or organs of the same animal may well produce very different results on the same substrate.

The pH value for optimum protease action was found to be 7.0 with more rapid decrease on the acid than on the alkaline side, as shown in Fig. 1. The action may be said to be tryptic in character.[11] The hydrogen ion concentration for the optimum action is very nearly that of the blood and probably of most of the tissues. As far as can be told from the results obtained, the kinetic relationships are similar to those observed by others as already pointed

[9] Cf. for example Wells, H. G., Chemical pathology, Philadelphia, 2nd edition, 1914, 456–8. Ewing, J., Neoplastic diseases, Philadelphia, 1919, 90–1.

[10] Abderhalden, E., and Medigreceanu, F., Z. physiol. Chem., 1910, lxvi 265. Abderhalden, E., and Pincussohn, L., Z. physiol. Chem., 1910, lxvi, 277.

[11] Cf. Loeper, G. F., and Tannet, G., Compt. rend. Soc. biol., 1920, lxxxiii, 993; J. pharm. et chim., 1920, xxii, 210.

out.   The facts that the substrate in such actions is not a simple one, and that probably several different reactions are occurring, either simultaneously or consecutively, serve to obscure the actual relationships of the actions.   In general terms, the actions are of the same type as other protease actions.

The relation between the protease actions and the growth of transplanted tumors may be summarized briefly.

The growth of the transplanted Flexner-Jobling rat carcinoma was followed, the tissue fragments before inoculation being immersed for various lengths of time in solutions of different hydrogen ion concentrations and salt contents.   The details of this work are presented elsewhere.[12]   The following conclusions are of interest in the present connection.   After immersion in phosphate mixtures of different hydrogen ion concentrations for 24 hours in the ice box, the transplants immersed in the pH 7.0 solutions grew normally, while those in the more acid and more alkaline solutions did not grow.   Immersion in physiological salt solution (0.15 M NaCl) at pH 7.0 for 24 hours did not prevent growth, with immersion for 72 hours, however, no growths were obtained. With calcium chloride solutions (0.078 M $CaCl_2$), growth was prevented in much shorter times of immersion, 10 hours or less.   The inactivating or harmful effects of the sodium chloride in the longer period of immersion, or of a much more dilute calcium chloride solution in a correspondingly longer time were overcome by the use of a "balanced" mixture of the two salts.   Thus, immersion in a solution of 0.15 M NaCl and 0.003 M $CaCl_2$ concentration for 72 hours showed normal growths of the transplants.

Summarizing the comparison of the protease actions of the aqueous extracts of human and rat tumors and the growth of the Flexner-Jobling rat carcinoma after immersion in different solutions before transplantation, it may be stated that the same optimum or favorable acidity of the solution is necessary for both (in the neighborhood of pH 7.0), that sodium chloride and calcium chloride exert retarding, inhibiting, or harmful actions on both sets of phenomena, calcium chloride much more so than sodium chloride for corresponding concentrations.   On the other hand, in the tumor transplants, antagonistic salt action was

[12] Sugiura, K., Noyes, H. M., and Falk, K. G., *J. Cancer Research*, 1921, vi, in press

observed with sodium chloride and calcium chloride, while in the
enzyme actions the salt effects were not antagonistic but appeared
to be additive.

The two sets of actions, although paralleling each other in certain respects, are evidently to be ascribed to different causes.
With the tumor transplants, the behaviors are connected with the
actions of the salts on the cell membranes and the changes in
permeability of the latter. With the enzyme actions, the effects
appear to be dependent upon a more direct interaction between
the salt and the enzymically active molecule or grouping. At the
same time, when it is considered that growth is connected with,
and possibly dependent upon, enzyme actions, even the partial
parallelism of the harmful effects of the different conditions upon
the cell membranes and upon the actions of the enzymes within
the cells, is of interest and perhaps of some significance.

SUMMARY.

The proteolytic actions on casein and on peptone of extracts
of malignant human tumors and of the Flexner-Jobling rat carcinoma were studied by the Van Slyke amino nitrogen and the
Sörensen formol methods at different hydrogen ion concentrations, in the presence of a number of neutral salts, for various
lengths of time, and with various concentrations of enzyme and
of substrate.

Similar results were obtained with the extracts from the two
sources. Optimum conditions for action were found at pH 7.0.
The general actions were similar to those of other protease preparations and could be formulated similarly. Tables and curves
showing the retardations exerted by various neutral salts and by
mixtures of several salts are given.

STUDIES OF URINARY ACIDITY.

I. SOME EFFECTS OF DRINKING LARGE AMOUNTS OF ORANGE JUICE AND SOUR MILK.

By N. R. BLATHERWICK and M. LOUISA LONG.

(*From the Chemical Laboratory of the Potter Metabolic Clinic, Santa Barbara Cottage Hospital, Santa Barbara.*)

It is known that the eating of most fruits leads to the formation of less acid urines due to the excess of base-forming elements in the fruits. The organic acids contained in the various fruits are oxidized within the body to carbon dioxide and water, so that the net result to the organism is a gain in base. However, fruits such as cranberries, prunes, and plums, which contain benzoic acid or its precursors, cause the production of more acid urines. This result is, doubtless, in part due to the synthesis of hippuric acid (Blatherwick, 1914).

During the progress of a study of the neutrality regulation of cattle, it was observed that a cow fed large amounts of corn silage, a product with an excess of base-forming elements, excreted an acid urine and showed a reduction in the plasma carbon dioxide-combining power (Blatherwick, 1920). The belief was then expressed that these effects were probably caused by the incomplete oxidation of the contained organic acids. The organic acids of corn silage are chiefly racemic lactic, acetic, and butyric, (Dox and Neidig, 1912, 1913). In view of the foregoing, the question arose as to whether the tolerance of man for naturally occurring fruit acids could also be passed. Orange juice was chosen for study because of its wide-spread use, and owing to the observation of Newburgh and Marsh (1920) that one of their diabetic patients succumbed after eating a bag of oranges.

Plan of Orange Juice Experiment.

Two healthy young women volunteered as subjects for this study. Subject O was given a uniform basal diet of the following composition; baked potato, 260 gm.; whole milk, 440 gm.; graham crackers, 300 gm.; raw apple, 150 gm.; cheese, 25 gm.; butter, 45 gm.; and egg, 65 gm.   The diet of Subject L was similar with the exception that she ate no egg.   After 4 days on this basal diet the effect of increasing amounts of strained orange juice was determined.

Methods.

The following analytical methods were used; pH, Palmer, Salvesen, and Jackson (1920–21); organic acids, Van Slyke and Palmer (1920); ammonia, Folin and Bell (1917); nitrogen, Folin and Wright (1919); and CO_2 capacity of plasma, Van Slyke (1917).

Discussion of Results with Orange Juice.

In Table I are shown the analytical data obtained from the the urines of the two subjects for the basal and experimental diets.   The effects on the urine of drinking large amounts of orange juice are the following: a marked increase in the pH value (less acidity); an increased excretion of organic acids; and a marked decrease in the ammonia output.   This increase in the organic acid excretion with a coincident decrease in the ammonia content is interesting because it illustrates an exception to the general rule of parallelism of these two factors.   The explanation of this peculiar result is probably the following: a certain part of the citric acid escapes oxidation and is eliminated in the urine as citrate and thus increases the titration value for organic acids, while the excess of base in the orange juice is sufficient to more than balance the organic acidity and to cause a marked depresssion in the ammonia excretion.   The values given by Sherman show that the ash of orange juice contains an excess of base amounting to 4.5 cc. of normal solution per 100 gm. of juice.   Therefore, on the last experimental day, the value for excess base from the orange juice, corresponded with 1,080 cc. of normal solution. Determination of the organic acid content of orange juice by the method of Van Slyke and Palmer indicated a concentration of 1.84 per cent citric acid.   Of this amount, 59 per cent appeared to be present as the free acid when titrated with 0.1 N NaOH,

Composition of Urine, and Plasma Carbon Dioxide Capacity.

Date	Urine					Plasma	Remarks
	Volume	pH	0.1 N organic acids	Ammonia N	N	CO₂ capacity	

Subject O.

Date	Volume	pH	0.1 N organic acids	Ammonia N	N	CO₂ capacity	Remarks
1921	cc.		cc.	gm.	gm.	cc.	
Nov. 4	875	6.8	507	0.26	7.79		Basal diet.
" 5	710	6.8	562	0.26	7.16		" "
" 6	720	6.6	522	0.25	7.06		" "
" 7	720	7.1	599	0.21	7.09		" "
" 8	1,355	7.1	777	0.19	8.35	63.3	Basal diet + 600 cc. orange juice.
" 9	1,750	7.1	647	0.16	7.35		" " +1,200 " " "
" 10	2,290	7.2	914	0.12	7.56		" " +1,800 " " "
" 11	3,160	7.4	929	0.13	8.21		" " +2,400 " " "
" 12						62.4	

Subject L.

Date	Volume	pH	0.1 N organic acids	Ammonia N	N	CO₂ capacity	Remarks
Nov. 4	1,210	6.4	488	0.21	6.07		Basal diet.
" 5	780	6.6	498	0.14	5.27		" "
" 6	610	6.9	512	0.13	5.98		" "
" 7	905	6.9	494	0.13	6.20		" "
" 8	1,700	6.9	585	0.15	6.29	59.5	Basal diet + 600 cc. orange juice.
" 9	2,250	7.3	699	0.10	5.67		" " +1,200 " " "
" 10	2,830	7.2	844	0.10	5.94		" " +1,800 " " "
" 11	2,550	7.4	878	0.07	6.98		" " +2,400 " " "
" 12						63.3	
						67.2	1 hour after drinking 800 cc. orange juice.

using phenolphthalein as an indicator. Since by this method citric acid is titrated to the extent of about 90 per cent, the true value for total citric acid would be about 2.0 per cent. The increased excretion of organic acids in the urines of the last day above the average excretion of the preliminary period amounted

to 382 and 380 cc. of 0.1 N acid. Assuming that these increases were entirely due to citric acid, an escape of 2.71 gm. of this acid into the urine is indicated. Therefore, approximately 6 per cent of the ingested citric acid appears to have escaped oxidation. Salant and Wise (1916) showed that after hypodermic injection of sodium citrate in rabbits about 12 per cent was excreted in the urine; with cats 30 per cent, and with dogs 40 per cent.

Observations of the plasma carbon dioxide capacity of Subject O showed practically no change as the result of drinking large amounts of orange juice. However, the plasma of Subject L gave an increase from 59 5 cc. before the orange juice period to 63.3 cc. at the close. On November 12th, this subject drank 800 cc. of orange juice at one time. Determinations of plasma CO_2 capacity showed values of 63.3 cc. before and of 67.2 cc. 1 hour afterward.

The foregoing results seem to render it improbable that the ingestion of large amounts of citrous fruits or fruit juices is capable of causing the production of an acid urine by overstepping the organism's ability to oxidize the organic acid or acids contained therein. (The results, of course, do not apply to fruits which yield acids other than citric in the body.) The subjects on the last day each drank 2,400 cc. or the equivalent of about 24 large oranges, containing approximately 48 gm. of citric acid. One may, therefore, feel secure in eating unlimited amounts of oranges without fear of acidotic effects.

Lactic Acid Experiments.

A basal diet of 2 liters of whole milk and 340 gm. of soda crackers was selected. This diet amply satisfies the ordinary requirements and is one which should theoretically yield a neutral urine. On the experimental days 2 liters of the lactic acid milk were substituted for the whole milk. This arrangement permitted a constant intake of the ash constituents throughout the experiment.

In addition to the determinations performed in the citric acid experiments, phosphorus was determined by the colorimetric method of Bell and Doisy (1920).

The experimental milk was prepared by inoculating skimmed milk with a lactic acid-producing culture and allowing it to ferment for 12 hours at 37 C. Such milk then showed an organic acid content of approximately

2 per cent, expressed as lactic acid, as determined by the method of Kramer and Greene (1921). The product at this point was full of large clumps of coagulated material which were next thoroughly broken up. Just before serving the calculated amount of cream was added to bring the fat content to that of whole milk.

RESULTS AND DISCUSSION.

Reference to Table II shows that the effects upon the urine of drinking large amounts of lactic acid milk were: a marked decrease in pH (increased acidity); a marked increase in the titratable acidity; a marked increase in the phosphorus content; a significant increase in the ammonia output; and no change in the organic acidity. These changes occurred in both subjects.

Determinations of the plasma CO_2 capacity led to rather variable results. Subject Ot. experienced no decrease in plasma CO_2 capacity upon changing from whole milk to sour milk but did show a marked decrease 1 hour after drinking 500 cc. of the acid milk. On the other hand, Subject Ol. responded with a decrease in the change from whole milk to the acid milk but showed no change as the result of drinking 500 cc. of this product. All in all, these findings may be taken as evidence that the ingestion of such large amounts of lactic acid are capable of reducing the alkaline reserve by the entrance of the acid into the blood. Our results are similar to those recently reported by Taistra (1921) who observed a decrease in plasma CO_2 capacity and an increase in the titratable acidity of the urine of a dog which was fed a meat broth containing lactic acid.

The mechanism involved in the production of the increased acidity of the urine resulting from the ingestion of lactic acid is very interesting. The increased output of acid in our experiments was caused by an augmented excretion of acid phosphate, as indicated by simultaneous increases in the titrable acidity and in the phosphorus content of the urines. None of the increased acidity was due to the organic acid fraction, that is, the lactic acid was completely oxidized or retained. The entrance of lactic acid into the blood stream seems to have evoked a compensatory elimination of acid phosphate by the kidney. The increased acidity of the urines resulting from the eating of fruits containing benzoic acid, and the strongly acid urines of diabetic patients may

perhaps be produced in a somewhat analagous manner. Further study along these lines is in progress.

TABLE II.

Composition of Urine, and Plasma Carbon Dioxide Capacity.

Date	Volume	pH	Titratable acidity	0.1 N organic acids	P	Ammonia N	Z	CO₂ capacity	Remarks
					P	Ammonia N	Z	CO₂ capacity	
1922	cc.		cc.	cc.	gm.	gm.	gm.	cc.	
Subject Ot.									
Jan. 23	1,480	6.5	218	453	0.77	0.39	12.64		Whole milk diet.
" 24	1,590	6.2	313	516	0.88	0.46	14.18		" " "
" 25	2,040	5.5	433	524	1.00	0.56	13.46	56.7	Acid milk diet.
" 26	2,270	5.7	391	547	1.06	0.60	13.03		" " "
" 27	1,830	6.5	200	457	0.84	0.42	11.87	57.6	Whole milk diet.
" 28								58.5	Before drinking 500 cc. acid milk.
								51.8	1 hour after drinking 500 cc. acid milk.
								59.5	3 hours after drinking 500 cc. acid milk.
Subject Ol.									
Jan. 23	1,480	6.9	121	501	0.65	0.43	11.01		Whole milk diet.
" 24	2,000	6.7	268	539	0.99	0.48	14.16		" " "
" 25	1,620	5.6	516	469	1.28	0.48	14.09	66.2	Acid milk diet.
" 26	2,225	5.9	444	554	1.22	0.56	14.11		" " "
" 27	2,090	6.8	220	557	1.00	0.46	13.65	59.5	Whole milk diet.
" 28								61.4	Before drinking 500 cc. acid milk.
								62.4	1 hour after drinking 500 cc. acid milk.
								62.4	3 hours after drinking 500 cc. acid milk.

SUMMARY.

The drinking of large amounts of orange juice resulted in the production of alkaline urines, an increased organic acid excretion,

and a decreased ammonia content of the urines.   It was impossible
to overreach the organism's ability to oxidize the contained citric
acid even though the amounts drunk in 1 day were the equivalent
of about 48 gm. of acid.

The drinking of large amounts of lactic acid milk caused the
formation of strongly acid urines.   This increased acidity was
shown to be due to the excretion of increased amounts of acid
phosphate, as indicated by simultaneous increases in titratable
acidity and phosphorus.   The lactic acid appeared to have been
completely oxidized or retained, as there was no change in the
organic acid excretion.

BIBLIOGRAPHY.

Bell, R. D., and Doisy, E. A., *J. Biol. Chem.*, 1920, xliv, 55.
Blatherwick, N. R., *Arch. Int. Med.*, 1914, xiv, 409.
Blatherwick, N. R., *J. Biol., Chem.*, 1920, xlii, 517.
Dox, A. W., and Neidig, R. E., *Iowa Agric. Exp. Station, Research Bull. 7*,
     1912; *Research Bull. 10*, 1913.
Folin, O., and Bell, R. D., *J. Biol. Chem.*, 1917, xxix, 329.
Folin, O., and Wright, L. E., *J. Biol. Chem.*, 1919, xxxviii, 461.
Kramer, B., and Greene, C. H., *J. Biol. Chem.*, 1921, xlvi, p. xxxviii.
Newburgh, L. H., and Marsh, P. L., *Arch. Int. Med.*, 1920, xxvi, 647.
Palmer, W. W., Salvesen, H., and Jackson, H., Jr., *J. Biol. Chem.*, 1920–21,
     xlv, 101.
Salant, W., and Wise, L. E., *J. Pharmacol. and Exp. Therap.*, 1916, viii, 123.
Taistra, S. A., *J. Biol. Chem.*, 1921, xlix, 479.
Van Slyke, D. D., *J. Biol. Chem.*, 1917, xxx, 347.
Van Slyke, D. D., and Palmer, W. W., *J. Biol. Chem.*, 1920, xli, 567.

THE FERMENTATION OF HEXOSES AND RELATED COMPOUNDS BY CERTAIN PENTOSE-FERMENTING BACTERIA.*

By W. H. PETERSON, E. B. FRED, and J. A. ANDERSON.

(*From the Departments of Agricultural Chemistry and Agricultural Bacteriology, University of Wisconsin, Madison.*)

The fermentation products of the hexoses usually differ from those of the pentoses. A common product, such as lactic acid, is generally produced from both types of sugar, but the difference usually lies in the other major products. The constancy with which lactic acid is formed from such different compounds as glucose and xylose indicates that three of the carbon atoms contained in the sugar molecules appear as lactic acid, while the residuum is converted into a two- or three-carbon compound depending upon the kind of sugar and the type of organism attacking it. In some cases this residuum is transformed into such products as acetic acid, ethyl alcohol, and carbon dioxide, singly or in pairs.

A cleavage of the molecules between the third and fourth carbon atoms counting from the alcohol end of the chain is indicated. The basis for this conclusion rests on the similarity of this portion of the structural formula of such different compounds as xylose, glucose, fructose, and mannitol, and the differences that obtain at the other end of their structural formulæ. A two-carbon residue, an aldehyde, a ketone, or an alcohol group is a potent factor in the determination of the end-products formed.

Cleavage along certain well defined lines, and constant intermediate products are indicated by such results. By a quantitative

111

determination of the end-products of fermentation and a study
of these data, it is possible that some rational theory such as exists
for the action of yeast, may be evolved concerning the mechanics
of carbohydrate fermentation by bacteria.

In previous publications (1, 2) we have described the end-products
formed from hexoses and related compounds by certain pentose-
fermenting bacteria which were designated as *Lactobacillus pen-
toaceticus*. From the aldo-hexoses, lactic acid, ethyl alcohol, and
carbon dioxide are formed approximately in the proportions re-
quired by the equation:

$$C_6H_{12}O_6 = C_3H_6O_3 + C_2H_5OH + CO_2$$

From fructose, acetic acid is formed instead of ethyl alcohol,
and as a corollary to the main fermentation, a reduction product,
mannitol, is produced. From the quantitative data, the following
equations may be deduced to represent the fermentation of
fructose:

(1) $C_6H_{12}O_6 + H_2O = C_3H_6O_3 + C_2H_4O_2 + CO_2 + 2H_2$

(2) $2C_6H_{12}C_6 + 2H_2 = 2C_6H_{14}O_6$

Two products of fermentation, carbon dioxide and mannitol,
are the most distinctive compounds for differentiating *Lactobacillus
pentoaceticus* from another group of pentose-fermenters which have
been described in a more recent publication (3). The latter form
no mannitol and produce only traces of carbon dioxide. Coinci-
dent with the absence of carbon dioxide is their failure to produce
ethyl alcohol or acetic acid from the hexoses. If the production
of ethyl alcohol and carbon dioxide be taken as indicative of the
enzyme, carboxylase, the first group of bacteria may be described
as carboxylase-positive and mannitol-forming, and the second
group as carboxylase-negative and non-mannitol-forming organ-
isms.

The members of the second group are in general somewhat more
vigorous fermenters, in that they attack a greater variety of
compounds, and destroy a larger percentage of the compound
attacked. They are less sensitive to changes in the reaction of
the medium, and operate through a wider range of hydrogen ion
concentration. The first group is inhibited by a slight alkalinity

while the second is able to grow in a fairly alkaline solution. The second group forms lactic acid from both halves of the sugar molecule, while the first group converts one-half of the molecule to ethyl alcohol (or acetic acid) and carbon dioxide. Lactic acid represents from 90 to 95 per cent of the sugar consumed by the second group, and accordingly the fermentation of the hexoses may be represented by the simple equation

$$C_6H_{12}O_6 = 2C_3H_6O_3$$

The disaccharide, lactose, and the trisaccharides, raffinose and melezitose, are fermented to the same end-product. In old cultures it is probable that a secondary fermentation of the lactic acid ensues with the production of acetic acid, traces of formic, and possibly ethyl alcohol and carbon dioxide.

The formation of lactic acid as the sole major end-product of fermentation relates these bacteria closely to the group of milk organisms designated *Streptococcus lactis* Lister. As a means of comparison, a representative of this group has been included in these experiments. A distinctive difference between the two, however, is the inability of the *Streptococcus lactis* group to ferment pentoses, which is the most conspicuous property of the organisms used for the fermentations discussed in this paper.

EXPERIMENTAL.

The kind of culture medium, the type of fermentation flask, and the methods of analysis for the different fermentation products have been described in previous papers (1). In most of the fermentations the acids formed were neutralized by the addition of sterilized 1.0 N sodium hydroxide. When the fermentable substance was not vigorously attacked as in the case of lactose and mannitol, better fermentation was obtained by keeping the solutions neutral with an excess of calcium carbonate. The improved results are no doubt due to a more favorable hydrogen ion concentration. The disadvantage in the use of calcium carbonate is that the carbon dioxide evolved by the bacteria cannot be determined when this salt is added to the medium.

Four cultures of pentose-fermenting bacteria, Nos. 29, 124-2, 102, and 31, and a strain of *Streptococcus lactis* have been used in

this work. Cultures 102 and 31 are of especial interest because they ferment arabinose, but do not attack xylose. Cultures 29 and 124-2 are much alike in their fermentative powers, but differ from Cultures 102 and 31 in that they are unable to ferment the trisaccharide, melezitose. *Streptococcus lactis* ferments neither melezitose nor raffinose.

Because of the labor involved and because certain of the sugars are not fermented, complete data for each sugar with all these bacteria are not given. It was decided to limit the fermentations to glucose, fructose, lactose, raffinose, and melezitose, as representative of the sugars, and to mannitol as representative of the hexahydric alcohols. Since the sugars used furnish all the monosaccharides obtained from maltose and sucrose it is felt that nothing of great importance would have been obtained by including these two disaccharides.

Fermentation Products.

The weight of sugar fermented, the quantity of products formed, and the relation of these products to the sugar consumed are given in Table I. The data show the extensive fermentation of all the sugars, practically complete in many cases and rarely less than 80 per cent. As a rule less than 0.1 gm. of sugar remained unfermented and sometimes as little as 0.03 gm. in 100 cc. of culture. As judged by the rate of acid production, Culture 124-2 was perhaps the most vigorous fermenter. The milk organism *Streptococcus lactis* was noticeably slower and less extensive than the pentose-fermenters; from 0.1 to 0.4 gm. of sugar remained unfermented in 100 cc. of culture.

Acids.—The sugars are fermented almost quantitatively to lactic acid, which product represents from 90 to 95 per cent of the sugar consumed. Volatile acid was not formed from the monosaccharides, but appreciable quantities were produced from lactose. Because of the slow rate of destruction of lactose, the fermentations were allowed to continue for from 40 to 60 days. No sugar was present at the end of this time, and it is probable that the bacteria were forced to use lactic acid as a source of energy. In the case of *Streptococcus lactis* no volatile acid was formed, due perhaps to the presence of sugar; 0.297 gm. remained even after 41 days. From the trisaccharides, only traces of

volatile acid were formed, possibly because of the difficulty with which these sugars are attacked, and as a consequence some of the lactic acid is converted into volatile acid. The production of volatile acids from lactic acid has been noted by several investigators (2, 4).

TABLE 1.

Total Fermentation Products from Sugars.

Culture No.	Age of culture.	Carbohydrate	Calculated for 100 cc of culture				
			Weight of compound fermented.	Volatile acid as acetic acid.	Non-volatile acid as lactic acid	Carbon dioxide	Weight of compound accounted for by products.
	days		gm	gm.	gm.	gm.	per cent
29	10	Glucose.	1.831	0.000	1.675	0.032	94
124–2	11	"	1.839	0.000	1.706	0.030	94
102	13	"	1.805	0.000	1.637	0.024	92
31	13	"	1.784	0.000	1.703	0.030	97
S. lactis.	15	"	1.790	0.000	1.599	0.020	91
29	10	Fructose.	1.620	0.000	1.434	0.023	90
124–2	10	"	1.635	0.000	1.510	0.019	95
102	8	"	1.620	0.000	1.520	0.026	95
31	8	"	1.586	0.000	1.434	0.030	92
29	15	Lactose.	1.335	0.000	1.211	0.045	91
29	63	"	2.036	0.078	1.807	Undetermined.	92
124–2	63	"	2.036	0.101	1.799	"	88
102	44	"	2.036	0.065	1.817	"	92
S. lactis.	44	"	1.785	0.000	1.578	"	86
29	24	Raffinose.	1.630	0.018	1.478	0.077	95
102	24	"	1.610	0.031	1.397	0.062	91
102	16	Melezitose.		0.010	1.641	0.049	
31	16	"		0.009	1.646	0.047	

It is noteworthy that the pentose-fermenters and the milk organism fermented the different sugars used in much the same way. The two types are, however, very different morphologically, and in their ability to ferment certain carbohydrates; the pentose-

fermenters attack a much larger number of compounds than *Streptococcus lactis*. In the cases where both ferment a given compound, the products seem to be alike and in about the same proportions. Another characteristic difference is the kind of lactic acid formed. The inactive form of lactic acid was produced by the pentose-fermenters while *Streptococcus lactis* always produced the active form.

Carbon Dioxide.—The carbon dioxide is too small to represent a direct fermentation product. From 20 to 80 mg. produced by the fermentation of approximately 2 gm. of sugar can scarcely be regarded as a fermentation product. It is more reasonable to assume this to represent the respiration of the cells. It may be regarded as a product of the endogenous metabolism of the cells; *i.e.*, the catabolic processes of cellular tissue. In this connection it should be noted that the amount of carbon dioxide produced from the pentoses, xylose and arabinose, by these organisms is essentially the same as that produced from the hexoses and their related compounds.

Fermentation of Mannitol.

The fermentation of mannitol is quite different from that of the sugars. These are either aldehydes or ketones while mannitol is an alcohol. No alcohol or volatile acid was obtained from the sugars while both of these products were found in the mannitol cultures. In every case ethyl alcohol was formed in the breaking down of mannitol, and in some of the fermentations in considerable amounts, from 8 to 16 per cent of the total end-products. Volatile acids which later will be shown to be formic and acetic were produced by all of the bacteria with the exception of No. 102. As in the case of alcohol, the volatile acids increase with the age of the culture. The lactic acid, on the other hand, decreases in the older cultures. These changes suggest a secondary fermentation of lactic acid to alcohol, formic acid, and acetic acid. Mazé (4) has shown that such a fermentation is possible. More recently Aubel (5) reported these same products in the fermentation of pyruvic acid. The rather difficult fermentation of mannitol forcing the organism to attack the lactic acid lends support to this hypothesis. On the other hand, the absence of alcohol from some of the sugar fermentations where lactic acid was apparently destroyed

is evidence against this view. A direct relation between alcohol and mannitol appears certain. The stereoisomeric structure of the compounds seems to determine the nature of the end-products. An alcohol group results in the formation of ethyl alcohol while an aldehyde or ketone group eventuates into lactic acid.

Certain differences in the fermentation products of the various bacteria manifest themselves. The pentose-fermenter, No. 102, never forms volatile acid while *Streptococcus lactis* forms conspicuously large amounts. Cultures 29 and 124-2 are large producers of both alcohol and volatile acid. The data are given in Table II.

TABLE II.

Total Fermentation Products from Mannitol.

Culture No.	Age of culture.	Volatile acid as acetic acid.	Non-volatile acid as lactic acid	Alcohol as ethyl	Carbon dioxide.
		gm	*gm*	*gm.*	*gm*
29	68	0 116	1 041	0 211	
29	38	0 000	1.209	0 011	
124-2	68	0 143	0.553	0 139	
124-2	38	0 085	0 991	0 129	
102	68	0 000	1 335	0 154	
102	38	0 000	0 364	0 064	
S. lactis.	68	0 182	0 336	0 042	
" "	38	0 070	0 427	0 047	
29	20	0 016	0 359	0 037	0 024
124-2	20	0 027	0 398	0 265	0 034
102	20	0 003	0 634	0 028	0 033
S. lactis.	20	0 130	0.438	0 052	0.034

(Columns 3–6 are headed: "Calculated for 100 cc. of culture.")

Forms of Lactic Acid Produced.

Many investigators (6) have determined the optical form of lactic acid produced by different bacteria. Levo, dextro, and inactive forms have all been produced by pure cultures. Some investigators have even claimed that the type of acid produced varies with the conditions of growth, but these claims rest on rather doubtful evidence.

In all of our work on pentose-fermenters, only inactive lactic acid has been found. The possibility of a small amount of an

active form has been considered but hitherto no attempt has been made to isolate such a form. To determine if any such form were present, the lactic acid obtained from glucose was subjected to fractional crystallization. As the active lactic acid is more soluble than the inactive type, fractional crystallization would separate the two salts. As a basis for comparison and as a check on the procedure, the lactic acid formed by *Streptococcus lactis* was included in the analysis.

The entire quantity of lactic acid produced by each culture was converted into zinc lactate and separated into three fractions. The different fractions were analyzed for their water of crystalli-

TABLE III

Water of Crystallization of Zinc Lactates Obtained from Glucose.

Culture No.	Crop No.	Weight of salt.	Loss on heating.	Water of crystallization.	Theory.
		gm.	*gm.*	*per cent*	*per cent*
124–2	1	3.3474	0.6032	18.02	18.1
124–2	2	0.5066	0.0884	17.47	18.1
124–2	3	0.1290	0.0234	18.14	18.1
29	1	1.9988	0.3618	18.10	18.1
29	2	0.8074	0.1440	17.83	18.1
29	2a*	0.7164	0.1294	18.06	18.1
S. lactis.	1	2.0970	0.2682	12.80	12.8
" "	2	0.3084	0.0396	12.84	12.8
" "	3	0.1284	0.0160	12.46	12.8

* Crop 2 recrystallized.

zation to determine if there was any departure from the theoretical amount. If both active and inactive zinc lactate were present, the inactive form would come out mainly in the first crop while the active form would appear largely in the last crop. No differences in the water of crystallization of the various fractions occurred, and all agreed well with the theoretical value.

The pentose-fermenters formed inactive lactic acid only, and *Streptococcus lactis* produced only the active form. The weights of zinc lactate and the loss of water on heating, together with the percentage of water of crystallization are given in Table III.

The water of crystallization for the zinc lactate produced from all the compounds fermented by the various bacteria are given

in Table IV. The blank spaces are due, either to the fact that a
fermentation was not made, as in the case of *Streptococcus lactis*
on fructose, or because the particular sugar is not fermentable by
the given bacteria; for example, Cultures 29 and 124-2 do not
attack melezitose. The pentose-fermenters produced inactive
lactic acid from all the different sugars and mannitol, while the
lactic acid organism from milk just as uniformly produced the
active enantiomorph.

The kind of active lactic acid produced by *Streptococcus lactis*
was ascertained by determining the specific rotation of the zinc

TABLE IV.

Forms of Lactic Acid Produced as Determined by the Water of Crystallization
of Their Zinc Salts. *

Source of lactic acid.	Crop No. of salt.	Culture 29	Culture 124–2	Culture 102.	Culture 31.	*S. lactis.*
		per cent	per cent	per cent	per cent	per cent
Glucose.	1	18.1	18.0	18.0	18.0	12.80
"	2	17.8	17.5	17.7	17.7	12.84
"	2a	18.1		18.2		
"	3		18.1			12.46
Fructose.	1	18.1	18.2	18.1	18.1	
Lactose.	1	18.0	18.1	18.1		12.9
Raffinose.	1	18.2		18.2		
Melezitose.	1			18.0	18.1	
"	2			17.9	17.9	
Mannitol.	1	18.0	18.2	18.2		13.1

* Inactive zinc lactate contains 18.17 per cent and active zinc lactate
12.8 per cent water of crystallization.

salts. The zinc lactate from lactose was recrystallized, and
1.2366 gm. of the anhydrous salt were dissolved in 30 cc. of water
at 22°C. The rotation produced at this temperature in a 2 dm.
tube was found to be −1.83° on the Ventzke scale. When cal-
culated to the corresponding specific rotation, the figure −7.67
was obtained. The value obtained in the same way for the zinc
lactate from the fermentation of glucose was −7.63. Hoppe-
Seyler and Araki (7), with comparable concentrations and tempera-
ture, found active zinc lactate to possess a specific rotation of
±7.522. Since the rotation of the zinc salt is the opposite of the
free acid, it is evident that this culture of *Streptococcus lactis*
produced *d*-lactic acid.

Identification of Volatile Acids and Alcohol.—The volatile acid
obtained directly from the culture and that resulting from the
oxidation of the alcohol were subjected to a Duclaux distillation,
and the distilling constants calculated from the titration data.
The constants obtained together with that for pure acetic acid,
are given in Table V. The constants for the acids resulting from
the oxidation of the alcohol from mannitol and volatile acid from
lactose are in good agreement in all cases with that for acetic acid,
and hence indicate that ethyl alcohol and acetic acid are produced
from mannitol and lactose, respectively. The constants for the
volatile acid from mannitol lie between those given by Duclaux
for acetic and formic acids. It is quite evident that the volatile
acid is a mixture. Since no qualitative tests indicated the pres-
ence of propionic acid, it was assumed that the mixture consisted
only of formic and acetic acids. From the distilling constants
the percentages of the two acids were calculated by the graphic
method of Gillespie and Walters (8) and are probably correct to
within 5 per cent.

The results were found to be as follows:

Culture No.	Formic acid.	Acetic acid.
	per cent	*per cent*
29	70	30
124-2	61	39
S. lactis.	50	50

As a check on the Duclaux data, the percentage of formic acid
was determined by Fincke's method (9). An immediate and heavy
precipitation of mercurous chloride left no doubt of the presence
of formic acid. The data are given in Table VI. A comparison
of the percentages of formic acid found by the two methods shows
good agreement in all but one case. There can be no question
that formic and acetic acids are produced in the fermentation of
mannitol by these different bacteria. It is not easy, however,
to visualize the mechanics of this fermentation whereby lactic acid
is the major product, and ethyl alcohol, formic acid, acetic acid,
and possibly small quantities of carbon dioxide, and even hydrogen
are minor products. With more extensive data it appears prob-
able that correlative processes of reduction and oxidation will
unfold the steps in the formation of these biochemical products.

TABLE V.

Distilling Constants of the Volatile Acids Obtained by the Duclaux Method.

Culture No.	Source of acid.	Fractions.									
		10 cc.	20 cc.	30 cc.	40 cc.	50 cc.	60 cc.	70 cc.	80 cc.	90 cc.	100 cc.
29	Lactose.	7.6	15.4	23.5	31.8	40.8	50.3	60.5	71.5	84.3	100
124-2	"	7.6	15.5	23.6	32.0	40.6	50.3	60.5	71.7	84.2	100
102	"	7.4	15.1	23.2	31.7	40.7	50.1	60.2	71.4	84.3	100
29	Mannitol.	6.2	13.2	20.4	28.1	36.5	45.6	55.8	67.5	81.5	100
124-2	"	6.7	13.9	21.4	29.2	37.7	46.8	56.8	68.2	81.8	100
102 *S. lactis*	"	6.7	13.8	21.6	29.6	38.1	47.3	57.3	68.9	82.2	100
29	Alcohol from mannitol.	7.7	15.8	23.0	32.8	42.0	51.6	61.8	72.9	85.3	100
124-2	" "	7.7	15.5	23.9	32.9	42.0	51.7	61.9	73.0	85.4	100
102	" "	7.6	15.7	24.1	32.8	41.9	51.5	61.8	72.9	85.1	100
Duclaux constant for acetic acid.		7.4	15.2	23.4	32.0	40.9	50.5	60.9	71.9	84.4	100

TABLE VI.

The Formic Acid Content of the Volatile Acids Produced from Mannitol.

Culture No	Total acid in aliquot as 0·1 N.	Weight of HgCl	Formic acid equivalent to HgCl	Percentage of formic in volatile acid.
	cc.	gm.	gm	per cent
29	23·6	0·6684	0·0652	60·0
124–2	29·5	0·8100	0·0790	59·0
S. lactis.	23·5	0·5854	0·0571	52·8

SUMMARY.

The fermentation products formed by this new group of pentose-fermenters from glucose, fructose, lactose, raffinose, and melezitose have been determined. All of these compounds are converted almost quantitatively into lactic acid. The lactic acid represents 90 per cent or more of the sugar fermented.

Glucose and fructose are almost entirely destroyed; in most cases, less than 0.1 gm. per 100 cc. of culture remained unfermented. Lactose, raffinose, and melezitose are less readily fermented; as much as 0.3 gm. per 100 cc. of culture may remain unfermented after 40 days. In 68 days all the sugar has been destroyed and a secondary fermentation of lactic acid begun. Volatile acid is formed in these old cultures.

Carbon dioxide is produced in small quantities, from 0.01 to 0.03 gm. per gm. of sugar destroyed. The amount is too small to represent a direct fermentation product. It is more plausible to regard it as a product of cell respiration. From these data it may be concluded that the bacteria produce no carboxylase.

The influence of a terminal alcohol group is manifested in the fermentation of mannitol. Ethyl alcohol, formic acid, and acetic acid are produced in addition to lactic acid. From 10 to 30 per cent of the total products is represented by these three compounds. It is suggested that these products are the result of correlative processes of reduction and oxidation.

The fermentation products of *Streptococcus lactis* like those of the pentose-fermenters are modified by the structural configuration of the fermented compound.

The pentose-fermenters produced only the inactive form of lactic acid while the active isomer was the form produced by the strain of *Streptococcus lactis*. Fractional crystallization failed to show any mixture of the two forms in any case.

BIBLIOGRAPHY.

1. Peterson, W. H., and Fred, E. B., *J. Biol. Chem.*, 1920, xli, 431.
2. Peterson, W. H., and Fred, E. B., *J. Biol. Chem.*, 1920, xlii, 273.
3. Fred, E. B., Peterson, W. H., and Anderson, J. A., *J. Biol. Chem.*, 1921, xlviii, 385.
4. Kayser, E., *Ann. Inst. Pasteur*, 1894, viii, 737.   Gayon, U., and Dubourg, E., *Ann. Inst. Pasteur*, 1901, xv, 527.   Mazé, P., *Compt. rend. Acad.*, 1913, clvi, 1101.   Mazé, P., and Ruot, M., *Compt. rend. Soc. biol.*, 1917, lxxx, 336.
5. Aubel, E., Thèse de Paris, Recherches biochimiques sur la nutrition du bacille pyocyanique, Caen, 1921, Series A, No. 887, Order 1702, 63.
6. For reviews of the literature see Currie, J. N., *J. Biol. Chem.*, 1911–12, x, 201.   Barthel, C., *Z. Gärungsphysiol.*, 1913, ii, 210.
7. Hoppe-Seyler, F., and Araki, T., *Z. physiol. Chem.*, 1895, xx, 371.
8. Gillespie, L. J., Walters, E. H., *J. Am. Chem. Soc.*, 1917, xxxix, 2027.
9. Fincke, H., *Biochem. Z.*, 1913, li, 253.

A STUDY OF THE ACETONE AND BUTYL ALCOHOL FERMENTATION OF VARIOUS CARBOHYDRATES.*

By GUY C. ROBINSON.

(*From the Department of Zymology, University of Toronto, Toronto, Canada.*)

A great deal of research has been performed on the acetone and butyl alcohol fermentation of maize, potatoes, horse-chestnuts, and other cereals rich in starch, in which various organisms of the widely distributed *Granulobacter* type have been employed. No systematic investigation has been made, however, of the action of this type of organism on other carbohydrates; namely, the various sugars, dextrins, and sugar alcohols. In consequence, at the suggestion of Professor Horace B. Speakman, a survey of these sources of carbon has been made with a freshly isolated soil organism. It was hoped that such an investigation would throw some light upon the biochemistry of the acetone and butyl alcohol fermentation by defining exactly which carbon compounds can be utilized by the organism.

In the early literature are found reports of work with various sugars but these are mainly concerned with the determination of end-products. Beijerinck (1) employed malt-wort in the butyl alcohol fermentation by means of *Granulobacter butylicum* and found maltose far better suited to the fermentation than glucose. Perdrix (2) submitted glucose, saccharose, and lactose to fermentation by an organism isolated from the Seine and called by him "*Bacillus amylozyme.*" The fermentation products from these sugars were acetic and butyric acids, hydrogen, and carbon dioxide, but no alcohols were formed. These latter products, however, were produced in considerable quantities from starchy

materials. Grimbert (3), on the other hand, in working with
Bacillus orthobutylicus, studied a large number of carbohydrates,
including glycerol, mannitol, glucose, invert sugar, saccharose,
maltose, lactose, arabinose, galactose, starch, dextrin, and inulin.
This investigator was interested mainly in the end-products of
the fermentation and did not study the course of the fermentation
in detail. Later, amylaceous material was used extensively in the
manufacture of acetone and butyl alcohol by this fermentation
process (4, 5). More recently, Speakman, and Reilly and co-
workers (6, 7, 8) have studied the biochemistry of the fermenta-
tion of starch by means of the Weizmann bacillus. In none of
this later work has the fermentation of sugars been studied,
hence the present investigation was thought advisable.

In this work the following carbohydrates were studied:

Monosaccharides.	Disaccharides.	Trisaccharides.	Polysaccharides.
Xylose.	Sucrose.	Raffinose.	Starch.
Arabinose.	Maltose.	Melezitose.	Dextrin.
Glucose.	Lactose.		Inulin.
Fructose.	Melibiose.		
Mannose.	Trehalose.		
Galactose.			

In addition the two alcohols, glycerol and mannitol, were investi-
gated. A number of sugar mixtures also were submitted to
fermentation in order to study the behavior of the organism with
respect to sugar preference, if any existed, when both mono- and
disaccharides were offered. In these following experiments as in
previous work (6), the course of the fermentation was followed by
determinations of the titratable acidity at periodic intervals.
Sugar determinations were likewise made throughout the fer-
mentation period in order to observe the progress of carbohydrate
consumption and to ascertain, if possible, the exact method by
which each compound was utilized by the organism.

The specific organism employed in the fermentations was a
pure culture of a *Granulobacter* type of organism originally iso-
lated from a sample of fresh barley which was grown in central
Pennsylvania during the summer of 1919. When first isolated,
the organism gave sluggish fermentations. However, as a result

of constant cultivation on laboratory media for nearly 3 years, an exceedingly hardy strain of the bacillus has been produced. It was with this strain that the present fermentations were performed.

Bacteriological Methods.

Preparation of Media.—After experimenting with a large number of well known mineral salt solutions, the following mixture was adopted as being the best suited for the fermentations at hand:

Monopotassium phosphate	1.00 gm.
Magnesium sulfate	0.20 "
Sodium chloride	0.01 "
Ferrous sulfate	0.01 "
Bacto-peptone	5.00 "
Distilled water	1,000 cc.

In the first part of the investigation sugar concentrations of 5 per cent were used but later it was discovered that more complete fermentations resulted with 3 per cent solutions; therefore, approximately this concentration was used in the subsequent experiments. In preparing the medium, in the main, 750 cc. of the above nutrient solution were placed in a liter experimental flask. These flasks were specially made Pyrex Erlenmeyers provided with short outlet tubes attached about an inch from the bottom. During use the sampling outlets were fitted with short pieces of good rubber tubing closed tightly with close-fitting screw-clamps. The weighed amount of sugar or other carbohydrate was then added to the liquid and the flask shaken until complete solution was effected. Thereafter, two 12 cm. filter papers cut in 1 cm. strips, were added, the flask was plugged well with cotton and tied over with waxed paper to minimize evaporation during the incubation period. The addition of the strips of filter paper is understood when it is recalled that the organism used is essentially an anaerobe, and that the paper provides excellent centers for the formation of gas bubbles, which soon develop at such a rapid rate that very shortly all of the oxygen is driven out of the medium. At the height of the fermentation the paper is completely buoyed up to the surface of the medium in the form of a matted head, held together by the slime formed, and supported

by the gas bubbles. Under such anaerobic conditions extremely active fermentations occur. The flask of medium as prepared above is then sterilized in the autoclave at 5 pounds pressure for 30 or 40 minutes.

Inoculation and Incubation.—The organism used is an anaerobic spore former, the spores of which are heat-resistant. Spore stocks were kept on hand and active cultures were prepared from these in maize mash tubes. Cultures 24 hours old were employed throughout, and approximately the same proportion of culture was used for the various flasks. About 20 cc. of inoculum were used for a flask containing 750 cc. of medium. Tubes and flasks were both incubated at 37°C. which is the optimum temperature for fermentation. Before removing samples for analysis, the contents of the flasks were always shaken well in order to obtain even distribution of head and clear liquid.

Sampling.—Suitable samples were periodically removed through the side outlet tubes by opening the screw-clamps and allowing the well mixed solution to run into a graduate cylinder. Usually 50 cc. were taken which served for both acidity and sugar determinations. After closing the clamp tightly, the end of the rubber tube was carefully swabbed out with cotton soaked in saturated aqueous carbolic acid solution. The opening was then plugged with a fresh saturated swab. With this technique no contamination was encountered.

In other experiments in which the rarer sugars were used, smaller amounts of medium were prepared in ordinary Erlenmeyer flasks of various sizes. Samples were removed from these by means of sterilized pipettes.

Chemical Methods.

Acidity.—Duplicate 10 cc. samples of the fermenting medium were titrated directly with 0.1 N sodium hydroxide, using phenolphthalein as indicator.

Sugar Determinations.—Two 10 cc. portions of the original sample were pipetted into two 15 cc. centrifuge tubes and to each were added 1 cc. of saturated normal lead acetate solution and 2 cc. of alumina cream. After mixing well by inverting several times the tubes were whirled in a centrifuge for 5 minutes. Thereafter 10 cc. of the clear supernatant solution were pipetted from

each tube and transferred to a 100 cc. volumetric flask. The sugar solution was then diluted to about 75 cc. and freed from lead by adding small quantities of anhydrous potassium oxalate. The volume was then completed to the mark, the solution well mixed, and finally filtered through a dry filter into a dry flask. The perfectly clear colorless solution was then used for the sugar determinations.

Determination of Reducing Sugars.—Two methods were employed in the investigation for the estimation of reducing sugars. The general method was that of Brown, Morris, and Millar (9), which is a carefully standardized modification of Fehling's method. Standard solutions were prepared as recommended by the authors and frequent blanks on the reagents were run, due account thereof being taken in all calculations. The same standard apparatus for heating, reducing, etc., was used throughout the investigation. It was found that this gravimetric method yields excellent results and, in truth, is much simpler to carry out than many of the more recent involved volumetric methods.

The second method of determining reducing sugars was a standardized modification of Barfoed's method, as recommended by Legrand (10). However, in it we employed a modified reagent which minimizes the hydrolysis of disaccharides, instead of the original Barfoed's solution. This method was used to estimate monosaccharides in the presence of reducing disaccharides such as maltose, lactose, and melibiose; and it was found with careful control to give fairly accurate results which were sufficiently reliable for our purpose. The new Barfoed reagent was prepared as follows:

Neutral normal copper acetate.........................	50 gm.
Sodium acetate..	50 "
Glacial acetic acid....................................	5 cc.
Distilled water to.....................................	1,000 "

By carefully standardizing the procedure good results were secured with known mixtures, and it was found that under the conditions noted below neither lactose nor maltose yielded any cuprous oxide whatever with the test. The procedure followed was as given below:

A special reduction vessel was made from a smooth thick-walled Pyrex Erlenmeyer flask of 150 cc. capacity by molding a lip on

one side of the neck so as to facilitate pouring and to serve as the
steam outlet. A cover was made from a small thistle tube by
cutting off and sealing the stem about ¾ of an inch from the bowl.
When this was placed in the neck of the flask only a small aperture
remained at the lip for the passage of steam and in this way
excessive loss of acetic acid was prevented. The test for mono-
saccharides was performed with 25 cc. of sugar solution and 25 cc.
of the reagent. An ordinary Bunsen burner was used for the
heating, the flame being closely guarded against draughts by a
shield. Before running the test with the sample the flame was
carefully adjusted with a blank solution consisting of 25 cc. of
Barfoed's solution and 25 cc. of water, so that the liquid reached
the boiling point in exactly 3 minutes. With each test the time
was noted and brisk boiling was allowed to proceed for exactly 3
minutes, at the end of which time the cuprous oxide was filtered
off immediately and washed thoroughly with at least 300 cc. of
boiling water. The crucible containing the oxide was next dried
for 2 hours at 100°C. in a hot water oven, cooled for 20 minutes in
a desiccator, and weighed.

It was found that glucose yielded approximately 1.61 times as
much cuprous oxide with Fehling's solution as with Barfoed's.
This factor, however, varies with the concentration of sugar, so
its application is limited. However, where the factor has been
used in the calculations it has been checked by determining the
copper values by both methods of known solutions containing
approximately the same concentrations of sugars as the samples.

EXPERIMENTAL.

The carbohydrates employed were either Difco or Pfanstiehl
preparations. Usually they were used as received without puri-
fication but in some cases recrystallization from alcohol of various
strengths was resorted to in order to obtain purer products. Such
was the case with glucose, maltose, and lactose.

Fermentation of Monosaccharides.

In Table I are given the acidity and sugar data of the fermenta-
tion of these sugars. It is noted that a high initial acidity occurs
in each case, due to the use of the particular acid medium which

contains dihydrogen phosphate. Examination of the table shows that these monosaccharides fall into two groups; viz., Group I consists of glucose, fructose, and mannose; and Group II. xylose and galactose. The sugars of Group I are all fermented normally as evidenced by the rise in acidity to a maximum, followed by a decline and later by a second slight increase at the end of the fermentation. These changes are more clearly shown in Fig. 1. Here it is also seen that the sugars of Group II behave differently; the galactose curve never falls after once reaching the maximum point of 6.2 cc. In a somewhat similar manner the xylose acidity reaches a very high maximum and falls only a very

TABLE I.

Fermentation of Monosaccharides.

After inocula-tion.	0.1 N NaOH per 10 cc. of medium.					Sugar per 100 cc. of medium.				
	Glu-cose.	Fruc-tose.	Man-nose.	Galac-tose.	Xylose	Glu-cose.	Fruc-tose.	Man-nose.	Galac-tose.	Xylose.
hrs.	cc.	cc.	cc.	cc.	cc.	gm.	gm.	gm.	gm.	gm.
0	1.30	1.12	1.28	0.91	1.00	3.82	3.54	2.76	2.88	2.55
6	1.90	2.56		2.02		3.81	3.39		2.77	
24	3.35	3.88	4.04	2.82	3.85	2.85	2.53	2.23	2.46	2.03
29	2.97	2.80	4.42	3.65	4.38	2.54		2.08		1.94
48	3.03	2.73	3.00	6.14	4.98	1.62	0.95	1.05	1.87	1.58
53	3.11		2.49	6.18	4.85	1.44		0.65		1.50
72	2.63	3.35	2.34	6.20	4.51	0.29	0.39	0.00	1.85	1.23
96	2.75					0.00	0.00			
120			2.23		3.87					
Sugar fermented, *per cent*						100	100	100	35	52

small amount, this occurring after the fermentation has practically run its course.

With respect to the percentages of sugar fermented there is likewise a marked difference in the action of the organism on these two groups of sugars. Whereas with glucose, fructose, and mannose the sugars are entirely removed from the medium, with the other two monosaccharides incomplete fermentations occur. About half of the xylose and only from 10 to 35 per cent of the galactose are utilized. In Fig. 2 the logarithms of the sugar concentrations at the different stages of the various fermentation periods are plotted, thus the curves indicate the different rates of

sugar consumption.   We see that in the first stage of the fermentation in each case, *i.e.* the stage of rapid acid formation during the

Fig. 1.   Acidity curves of the monosaccharide fermentations.

Fig. 2.   Sugar curves of the monosaccharide fermentations showing the different rates of sugar consumption.

first 30 or 36 hours, the rates of fermentation of the various sugars are practically identical.   Beyond this stage the curves show

striking differences and the sugars again fall into the same two groups. It is interesting to call to mind that these first three sugars, namely glucose, fructose, and mannose, are interconvertible into one another when in solution in the presence of a trace of alkali, through the formation of a common enolic form. Galactose, however, does not enter the enolic system and it is this sugar which does not ferment normally. The action of our organism on these particular sugars to a certain extent resembles that of ordinary yeast, except that xylose is partially fermented in the present case. In this connection it is interesting to point out the difference between the bacillus here studied and the lactobacillus of Peterson and Fred (11), which ferments glucose and galactose with equal ease but only consumes from 30 to 45 per cent of mannose even after 38 days incubation.

In addition to the above fermentations of monosaccharides, a few small ones were performed with the pentose sugar, arabinose, and with the methyl pentose, rhamnose. The former sugar is fermented in a similar manner to xylose. Rhamnose, however, is not attacked at all.

Fermentation of Disaccharides.

Sucrose, Maltose, and Lactose. — We shall first consider the commoner sugars, sucrose, maltose, and lactose, as they have been investigated in greater detail than the rarer disaccharides. The results of these fermentations are given in Table II, and it is readily seen that all of these sugars ferment normally with respect to acidity formation and sugar consumption. With sucrose, however, it is observed that all the sugar had not disappeared within the usual time. This is mainly due to the fact that a much more concentrated medium was employed, and also, owing to the initial presence of invert sugar, to a delay in the fermentation of the sucrose itself. These phenomena will be considered in more detail when the fermentation of mixtures of carbohydrates is discussed. Other sucrose experiments, in which 3 per cent sugar was used, have shown that this sugar is completely fermented but in a slightly longer time whenever an appreciable amount of invert sugar is present. Upon analyzing the fermented sucrose solution for sugar at the end of 144 hours fermentation, there was found only a trace of reducing material present after hydrolysis with

acid.   All three of the common disaccharides are, therefore, completely fermented.

TABLE 11

Fermentation of Disaccharides.

After inoculation.	0.1 N NaOH per 10 cc. of medium.			Sugar per 100 cc. of medium.			
	Sucrose	Maltose.	Lactose	Sucrose.		Maltose.	Lactose.
				Invert sugar	Sucrose.		
Hrs	cc.	cc.	cc	gm	gm	gm	gm.
0	1.02	1.00	0.94	0.258	+.46	2.99	2.74
6	1.81	1.22	1.68	0.201	+.22	2.78	2.71
24	4.93	5.14	4.36	0.000	3.91	1.15	2.21
29	4.75	5.43	4.68		3.52	1.07	2.05
48	2.31	2.91	4.04		2.57	0.34	1.47
53	2.43	3.10			2.30	0.32	
72	2.96	3.19	2.92		1.46	0.00	0.51
77	3.08				1.31		
96			2.35		*		0.00
Sugar fermented, *per cent*..........				100		100	100

* A trace of sucrose remained after 144 hours of fermentation.

Fig. 3.   Acidity curves of the disaccharide fermentations.

Fig. 3 shows the acidity changes which take place during the fermentation of these sugars.   There is great similarity between the acidity curves of sucrose and maltose.   Although the acidity

of the latter sugar reaches a higher maximum, the falls in each case are parallel and of approximately equal amounts. The lactose acidity curve, on the other hand, differs from these somewhat, it being more drawn out with a less rapid fall after the maximum is reached.

Passing now to the sugar curves as plotted in Fig. 4, we find that sucrose and lactose ferment at approximately the same rate, whereas maltose is attacked much more rapidly. Furthermore, starting with practically equal concentrations of maltose and lactose, the sugar in the former flask is completely consumed fully 24 hours sooner than that in the latter. With respect to the

Fig. 4. Sugar curves of the disaccharide fermentations showing the different rates of sugar consumption.

fermentation of sucrose it should be mentioned that after the initial reducing sugar, present in the medium as a result of sterilization, had disappeared during the first few hours, at no time was any invert sugar observed during the whole fermentation period. Further consideration of this fact will be made later in this paper.

Melibiose and Trehalose.—In addition to the above common disaccharides small experiments were made with the two rare sugars, trehalose and melibiose. Trehalose failed to show the least sign of fermentation even after 2 weeks incubation, consequently it was concluded that the butyl bacillus is unable to split this sugar. Hence the organism does not contain trehalase.

This enzyme is contained, however, in certain fungi, especially *Aspergillus niger*, and many species of yeast, but according to Lafar no definite proof of its existence in bacteria has been brought forth. Fischer (12) contended that trehalase was identical with maltase but this is scarcely likely since the butyl organism here studied contains maltase but still does not hydrolyze trehalose.

A small quantity of melibiose was prepared according to the method of Bau (13), by hydrolyzing raffinose with 2 per cent acetic acid and fermenting out the fructose from the resulting mixture by means of a pure culture of top yeast. After purification with vegetable carbon, the remaining sugar solution, after adding the usual mineral salts and peptone, was inoculated with a butyl culture. For the first 10 hours active gassing occurred, but after this time the fermentation gradually subsided until at 24 hours no gas whatever was being evolved. The rise in acidity only amounted to 0.25 cc. of 0.1 N acid and the reducing sugars had only decreased a very small amount. The flask, however, was kept in the incubator for over a week but still no indication of a revived fermentation was observed, hence it was concluded that the initial gas production was due to the inoculum and to the destruction of the small amount of fructose left in the solution by the yeast. It is therefore concluded that melibiose alone is not fermented by the butyl bacillus.

Fermentation of Sugar Mixtures.

This series of experiments in which glucose was used in conjunction with other sugars was performed, first, for the purpose of finding out whether the butyl bacillus exhibited any preference for the simpler sugars, and secondly, for the purpose of determining whether in mixtures either sugar exerted any influence upon the fermentation of the other. The experiments were suggested by the peculiar behavior of the sucrose fermentation in the presence of an appreciable amount of invert sugar.

Glucose-Sucrose Mixture.—A sugar mixture consisting of 30 gm. of sucrose and 20 gm. per liter of medium was prepared and inoculated. The course of the fermentation was followed by the acidity readings and the consumption of the individual sugars was followed by periodic reducing sugar determinations both before and

after inversion. An active fermentation ensued immediately, the acidity attaining a maximum in about 30 hours. This was followed by the characteristic fall, and later by a marked rise which suggests a secondary fermentation. In Table III are given the results of this experiment as well as those of the maltose and lactose mixture fermentations which will be considered later. The sugar table of the glucose-sucrose experiment very clearly indicates that a

TABLE III.

Fermentation of Sugar Mixtures.

After inocula-tion.	0.1 N NaOH per 10 cc. of medium.			Sugar per 100 cc. of medium.					
	Glucose plus			Glucose plus					
				Sucrose.		Maltose.		Lactose.	
	Sucrose.	Maltose.	Lactose.	Glucose	Sucrose.	Glucose.	Mal-tose.	Glu-cose.	Lac-tose.
hrs.	cc.	cc.	cc.	gm.	gm	gm.	gm.	gm	gm.
0	0.96	0.99	0.97	2.08	2.65	0.68	2.30	0.68	2.19
6		1.92	1.39					0.53	2.17
				(At 20 hrs.)					
12		4.18	3.44	1.70	2.65	0.21	1.59	0.25	
24	3.70	4.83	4.88	1.63	2.64	0.13	0.85	0.00	2.22*
				(At 44 hrs.)					
29	4.20	4.22	5.10	1.34	2.64	0.00	0.63		
48	3.13	2.96	4.16	1.24	2.54	0.00	0.25	0.00	1.59
68				0.37	2.59				
72	3.12	2.96	2.66	0.23	2.62	0.00	0.00	0.00	0.14
96			2.35						0.00
116	3.19			0.00	2.28				
120				0.00					
140	3.66			0.00	1.82				
214				0.00	Trace.				

* High value due to error in the determination.

marked preference is shown by the bacillus for glucose as compared with sucrose when the two sugars are present in the medium. We see that 72 hours were required for the removal of the glucose and that during that time no sucrose was utilized. After this occurred there was a gradual destruction of sucrose until it was all consumed. During this secondary fermentation there was no observable invert sugar present in the medium. This either

means that there is no exocellular sucrase or else that the invert sugar, if formed, is destroyed as rapidly as it is produced. This point will be considered later in the paper. The data from this interesting experiment are plotted in Fig. 5 which shows very clearly how the sucrose persists until after the glucose has disappeared. Owing to lack of space the complete sucrose and acidity curves are not shown, nevertheless the details are given in the table. Normally the fermentation of glucose or sucrose requires about 3 or 4 days but this double fermentation lasted for fully 9 days.

Glucose-Maltose Mixture.—This fermentation was performed similarly to the one just described except that a lower concentra-

Fig. 5. Acidity and sugar curves of the fermentation of a glucose-sucrose mixture.

tion of total sugar and a somewhat different ratio of glucose to maltose were employed. The mixture consisted of 18 gm. of recrystallized maltose and 4.5 gm. of purified glucose dissolved in 750 cc. of nutrient solution. After inoculation the course of the fermentation was followed as before. However, in the present case, since maltose is a reducing sugar, it became necessary to employ Barfoed's method for the determination of the glucose in the mixture.

The acidity, as shown in Table III, rises very rapidly and then falls as in a typical pure maltose fermentation, differing in this respect from the glucose-sucrose mixture which gave a curve similar to that of a pure glucose fermentation. Also the sugar

data show that the amount of maltose starts to diminish at the very beginning as does also the glucose. However, since glucose is present in lower concentration it is consumed sooner than the maltose, but the rates of fermentation are approximately the same. Fig. 6 shows the sugar and acidity curves of the experiment. It is observed that these represent an entirely different type of fermentation than the one previously described. The organism does not show any preference for glucose or maltose. The question therefore arises: Does the butyl bacillus utilize the more complex maltose as such in the same way that it does the glucose, or is there an active maltase formed which immediately

Fig. 6. Acidity and sugar curves of the fermentation of a glucose-maltose mixture.

attacks the maltose, hydrolyzing it into the simpler glucose which is then in turn fermented? This question will be considered later.

Glucose-Lactose Mixture.—A mixture of 4.5 gm. of pure glucose and 18 gm. of lactose was fermented in a manner similar to the two mixtures just considered. The data given in Table III indicate that this fermentation resembles the glucose-sucrose fermentation in that the disaccharide is not fermented until the last trace of glucose has disappeared. After this has taken place, the lactose is then gradually utilized until at 96 hours no more sugar remains in the medium. The short time in which the fermentation was completed was due in part to the lower concentration of sugars employed.

Glucose-Galactose Mixture.— Having observed in the experiments with the monosaccharides that glucose is completely fermented and that galactose is less than a third destroyed, and also in the lactose experiments that this sugar is completely fermented, quite likely after hydrolysis, it seemed possible that the presence of a readily fermented sugar, such a glucose might exert some accelerating influence upon the fermentation of galactose. If lactose is hydrolyzed it follows that this would result in furnishing the organism with a mixture of equal parts of glucose and galactose. Furthermore, since we find no trace of sugar at the end of the fermentation we must conclude that both these sugars are completely fermented under these conditions. From this it would seem that glucose does exert some beneficial influence upon the action of the organism on the difficultly fermentable galactose. In order to discover whether such is actually the case the following experiment was devised:

In each of three experimental flasks were placed 750 cc. of nutrient solution and to the first were added 20 gm. of glucose, to the second 20 gm. of galactose, and to the third 10 gm. of each of these two sugars. After sterilization the flasks were inoculated with similar cultures. Acidity readings were made at regular intervals during the fermentations but sugar determinations were made only at the start and at the finish. The data of these experiments are given in Table IV. The glucose and galactose fermentations were of the same type as previously described, that is, the acidity of the former rose to a maximum and then fell while that of the latter maintained its high value throughout the course of the fermentation. However, the data of the mixed fermentation show interesting differences. In the first place the acidity is seen to rise sharply until a high value is reached, which is followed by a fall as in a pure glucose fermentation. Later a second rise is noted until a second high value is attained, which is considerably higher than the first. This acidity is then maintained as in a pure galactose fermentation. Considering only the acidity curves, which are plotted in Fig. 7, one can see that the mixed fermentation consists of two separate and distinct fermentations, one displaying the characteristics of a pure glucose fermentation and the other exhibiting those of a pure galactose fermentation. The first fermentation is finished in the first 36

hours and the second is practically complete in a further similar period.

TABLE IV.

Fermentation of Glucose, Galactose, and a Glucose-Galactose Mixture.

After inoculation.	0 1 N NaOH per 10 cc. of medium.			Sugar per 100 cc. of medium.		
	Glucose.	Galactose.	Ratio 1:1 Glucose-galactose.	Glucose.	Galactose.	Ratio 1:1. Glucose-galactose.
hrs.	cc.	cc.	cc.	gm.	gm.	gm.
0	1.01	1.02	0 95	2.54	2 0S	2 31
6	1.59	1.59	1 S2			
11	3.45	3.36	3.72			
24	4.12	5.19	4.1S			
30	3.74	5.4S	3 54			
48	2 S1	5.92	3 SS			
72	2.53	6.02	4 92			
96	2.55	6.04	4.91	0 00	1.97	0.55
Sugar fermented, *per cent*..........				100	10	Glucose ...100 Galactose.. 47

Fig. 7. Curves showing the comparison between the pure glucose, the pure galactose, and the mixed glucose-galactose fermentations.

We find that the total reducing sugar value has fallen from an initial concentration of 2.31 gm. per 100 cc. to a concentration of

0.55 gm. per 100 cc. (calculated as galactose). This shows a total loss of sugar of about 76 per cent. However, upon testing the resulting beer of the fermentation with the osazone test, using phenylhydrazine hydrochloride and sodium acetate, no glucosazone was detected, but large quantities of galactosazone were obtained. Assuming, therefore, that all of the glucose had disappeared from the medium, we find that a little over half of the galactose was fermented whereas with the pure galactose fermentation only about 10 per cent was consumed.

Fermentation of Trisaccharides.

Raffinose. The trisaccharide, raffinose, is completely fermented by bottom yeast owing to the presence of both invertase and melibiase. Top yeast, on the other hand, only partially ferments it, leaving as a residue the disaccharide, melibiose, because of the fact that these particular types of yeast do not contain the enzyme, melibiase. A number of molds and bacteria also ferment this trisaccharide, either partially or completely.

The medium for the raffinose fermentation was prepared by dissolving 9 gm. of the sugar in 350 cc. of nutrient solution and sterilizing at 5 pounds pressure for half an hour. After inoculation periodic samples were withdrawn by means of sterilized pipettes. These portions were analyzed for acidity, monosaccharides, and disaccharides. Use was made of both the Barfoed and the Brown, Morris, and Millar methods. By studying the acidity data given in Table V, it is observed that an abnormal fermentation takes place which is almost identical with that of the galactose experiments. The initial rise in acidity, however, is much slower in the case of raffinose and it reaches a lower maximum. Fig. 8 shows the curve based on these acidity data.

We will now consider the possible ways in which this trisaccharide can be attacked by any fermenting mechanism. Raffinose is composed of three monosaccharide molecules; namely, glucose, fructose, and galactose. Different enzymes have different actions upon the sugar, splitting it in different ways. There are, therefore, four possible ways by which raffinose can be attacked; namely, fermented (1) directly without preliminary hydrolysis; (2) after complete hydrolysis by the enzyme, raffinase;

(3) after the complete hydrolysis by the two enzymes emulsin and sucrase; and (4) after partial hydrolysis by sucrase, yielding

TABLE V.

Acidities of the Raffinose, Dextrin, and Starch Fermentations.

After inoculation.	0 1 N NaOH per 10 cc. of medium.			
	Raffinose.	Dextrin.		Starch.
		Acid hydrolyzed.	Amylase hydrolyzed.	
hrs	cc.	cc.	cc.	cc.
0	0 91	1 02	1 09	0 91
6	1 53	1 40	1 68	1 78
24	3.02	4 38	4 57	4 54
29	3.32	4.77	4.93	4.50
48	4.17	5.01	4 32	3.23
53	4 38	4 96	4 24	3 09
72	4 58	5.16	3 74	3.20
96		5.08	3.47	
120	4.62			

TABLE VI.

Sugar Data of the Raffinose, Dextrin, and Starch Fermentations.

After inoculation.	Sugar per 100 cc. of medium.				
	Raffinose.		Dextrin.		Starch.
	Fructose.	Melibiose.	Acid hydrolyzed.	Amylase hydrolyzed	
hrs.	gm.	gm.	gm.	gm.	gm.
0	0.00	0 00	0.00	0.078	0.00
6	0.00	0.00	0.091	0.083	0.18
24		0.141	0.208	0.312	0.66
29	0.294				0.71
48	0.361	0.368	0.104	0.156	0.74
53	0.406	0.356			0.72
72	0.450	0.382	0.109	0.052	0.47
84					0.25

fructose and melibiose. Of these four possible methods of attack, the first would appear to be highly improbable when we consider the method by which the disaccharides are utilized. The data

given in Table VII eliminates the second possible method because the presence of a disaccharide is clearly indicated by the wide differences between the successive Barfoed and Fehling values of the fermenting solution. To confirm this a mixture of equal proportions of glucose, fructose, and galactose, which by the way would result in case raffinose was completely hydrolyzed by raffinase, was tested for reducing values by both methods and in no case gave such wide differences. It is, therefore, quite evident that some sugar is formed during the fermentation which reduces Fehling's solution and does not reduce Barfoed's. This fact also eliminates Method 3, otherwise sucrose, a non-reducing sugar,

FIG. 8. Acidity and sugar curves of the raffinose fermentation.

would first form and then be split up by sucrase which is known to be present. It follows, therefore, that the butyl organism studied attacks raffinose by Method 4; that is, by splitting it into fructose and melibiose by means of sucrase.

In order to confirm this fact the osazone test was again resorted to for demonstration of the presence of melibiose and fructose in the fermented residue. Two osazones were prepared which after fractionation and repeated recrystallization from boiling water and 50 per cent alcohol, proved to be those of melibiose and fructose. The melibiosazone crystallized in fine, delicate medallions, having sharp saber-like points radiating from the center. These crystals melted from 180–182°C. which agrees closely to Browne's

(14) figure of 179° for melibiosazone. The other compound resembled the osazone of glucose, fructose, and mannose, in structure, and melted at 205°C. It is no doubt formed from fructose since this sugar is the one produced from raffinose when melibiose is split off.

Since fructose and melibiose are present in the fermented beer it follows that these sugars are formed during the fermentation by the action of the enzyme, sucrase, which is present in the butyl bacillus. This enzyme is not secreted, however, because in the presence of toluene no reducing sugars are produced in a pure raffinose medium inoculated with a large volume of active culture. Hence the enzyme must function within the cell. In view of this fact it is supposed, in considering the mechanism of the raffinose fermentation, that the sugar enters the cell and is there hydrolyzed

TABLE VII.

Raffinose Fermentation.

After inoculation.	Cuprous oxide per 25 cc. of solution.		
	Barfoed's value.	Fehling's equivalent	Fehling's value.
hrs.	*mg.*	*mg.*	*mg.*
29	14.8	23.8	
48	18.0	29.0	47.5
53	20.1	32.4	50.3
72	22.3	35 9	55.1

into fructose and melibiose. Fructose, being readily fermented is at first consumed, but melibiose not being so easily fermented accumulates after a short time in the cell. It is due to the active destruction of fructose that vigorous gassing and a rise in acidity occur. After a certain amount of melibiose has accumulated in the cell it diffuses out into the medium and is there detected by Fehling's solution. Eventually after about 24 hours some unknown inhibiting factor or combination of factors begins to interfere and the fermentation is retarded. This is accompanied by the slowing up of gas production and a slackening up of the acid formation. Beyond this point fructose begins to accumulate and, consequently, diffuses from the cell into the medium where its presence is detected by the Barfoed test. Finally the inhibiting influence becomes so effective that the fermentation stops alto-

inoculation with 10 cc. of active culture the acidity and reducing values of each fermenting mixture were followed periodically. The acidity data are given in Table V. The figures show that the two fermentations are not at all alike. With the biological product a perfectly normal fermentation occurs, but in the case of the acid-hydrolyzed dextrin the fermentation is quite abnormal, comparing well with that of galactose. These data are plotted in Fig. 9 as Curves I and II, of which Curve I represents the acidity changes occurring in the fermentation of the acid product, and Curve II those of the amylase product. The former curve is of the same type as the galactose acidity curve while the latter resembles the curve of a lactose fermentation. The reducing sugar data of both fermentations are found in Table VI and are plotted in Fig. 9 as Curves I and II. These two curves are similar to the sugar curve of the starch fermentation. In order to determine the extent to which each type of dextrin was consumed, samples of the fermented solution were filtered and aliquots analyzed for residual dextrin by completely hydrolyzing with hydrochloric acid and determining the glucose formed. It was found that with the acid product 3.26 gm. of dextrin remained in the solution indicating a consumption of about 60 per cent. The amylase product was completely fermented. This seems to indicate a marked difference in the character of the two dextrins prepared by different methods, one chemical and the other biological. In this connection it is interesting to draw attention to the fact that dextrins prepared by the action of malt diastase are much more easily hydrolyzed by pancreatic juice than are dextrins which are prepared by the acid hydrolysis of starch (15). However, in the present case, until further evidence is secured by additional experiments, it cannot be definitely stated that the difference between the availabilities of the two dextrins here studied is entirely due to the modes of preparation or to the fact that in the one case erythrodextrin was used and in the other the achroodextrin was employed. Further study will be undertaken in the near future on this point as it appears to be of more than passing interest.

Inulin.— A small flask of inulin was fermented and the course was followed by the acidity determinations. Initial and final inulin values were also determined so as to discover to what extent

t carbohydrate was consumed. The results of the acidity
t ations are given in Table VIII and the curve of the same is
f nd in Fig. 10. In this case also an incomplete fermen-
t ion is encountered, as shown by the maintained high
a lity readings. This fact was likewise borne out by the inulin

TABLE VIII.

Acidities of the Melezitose, Inulin, and Mannitol Fermentations.

fter inoculation.	0 1 x NaOH per 10 cc. of medium.		
	Melezitose.	Inulin.	Mannitol.
hrs.	cc.	cc.	cc.
0	1 01	0.96	1 02
24	4.1S	3.47	3.36
30	4.41	4.23	4 02
4S	5 05	4 36	4 S3
54	5.00	4 3S	4.93
72	5.05	4.47	5.05
120	5.06	4 47	4.S6

FIG. 10. Acidity curves of the melezitose, inulin, and mannitol fermentations.

d erminations, performed by first hydrolyzing the solutions with
h lrochloric acid and then determining the fructose formed.
S ghtly less than half of the inulin was consumed by the bacillus.
D ring the fermentation period no reducing sugar appeared in the
m dium until near the end of the fermentation.

Fermentation of Alcohols.

Mannitol. The medium used in this experiment consisted of a 3 per cent pure mannitol solution with the usual nutrient salts added in the correct proportions. Upon inoculation this medium was found to ferment vigorously with extremely active gas production and a rapid rise in acidity. This fermentation proved to be one of the abnormal type as shown by the maintained high acid value after the maximum was reached. The acidity data are plotted in Fig. 10. No data was obtained showing the extent to which this carbohydrate was fermented by the organism.

Glycerol.—A medium containing 3 per cent of pure glycerol was prepared and inoculated with active culture. After the carbohydrate contained in the added inoculum was consumed there was no evidence whatever of any fermentation of the glycerol. Hence it is concluded that this material is not available as a source of carbon for the butyl bacillus.

Hydrolysis of Carbohydrates in the Presence of Toluene.

Knowing from previous experiments that the organism is capable of utilizing such carbohydrates as sucrose, maltose, lactose, raffinose, melezitose, dextrin, starch, and inulin, as well as the simpler monosaccharides, the question at once arose as to the exact manner in which these compounds were attacked. In the case of colloidal substances such as starch, dextrin, and inulin, it is obvious that the organism must first effect hydrolysis before these materials can enter the cell. Hence it follows that the bacillus must secrete exocellular enzymes to effect these hydrolyses. Sugars, however, are capable of passing through the membrane with facility, and initial hydrolysis is not therefore essential. For each sugar there are three possible methods of attack; *viz.*, (1) direct utilization without preliminary hydrolysis; (2) preliminary hydrolysis by an exocellular enzyme; (3) hydrolysis within the cell by an enzyme not secreted. If the first of these methods obtains we must assume the presence within the cell of an elaborate enzyme system capable of converting a large number of sugars of very different structure into common end-products. On the other hand, it seems more probable that the di- and trisaccharides are first split into their respective simple hexoses either without

within the cell, and that these sugars are then attacked by the
acid-forming enzymes. The raffinose experiments have demon-
strated in a definite manner the existence of sucrase. Assuming
that lactase and maltase also exist, our conception of the enzyme
system becomes much simpler. Any sugar, for instance, which,
following hydrolysis, yields the hexoses, glucose, fructose, or man-
nose, can be fermented by the same enzyme system owing to the
fact that these sugars are interchangeable, passing through a
common enolic form when in solution. Galactose, however,
when produced by hydrolysis, requires a special mode of attack,
since this hexose does not pass into the same enolic form as do the
other three sugars mentioned. Nevertheless, this special galacto-
enzyme, which is required, exists in the butyl organism because
we find that pure galactose is at least partially fermented and
lactose completely.

Since there is little doubt but that the complex sugars are first
hydrolyzed before they are fermented, the question still remains
as to whether these hydrolyses take place outside or inside the cell.
The problem is considered in the following series of experiments.
The method of study, briefly stated, consisted of preparing
flasks of the various carbohydrates; inoculating these with active
cultures; incubating for about 24 hours; and finally adding toluene
to stop further cell activity. Changes in the reducing sugar
values were then noted and compared with suitable controls in
order to determine whether any hydrolytic action had taken
place by secreted enzymes.

Sucrose, Maltose, and Lactose.—To 300 cc. flasks, each contain-
ing 200 cc. of nutrient solution, were added 6 gm. of the sugar to
be tested. The solutions were sterilized at 5 pounds pressure for
$\frac{1}{2}$ hour. No hydrolysis occurred in the sucrose flask and only a
slight amount in those containing maltose and lactose. Each
flask was inoculated with 10 cc. of active culture and incubated at
3°C. for 26 hours. To 100 cc. portions of the actively fermenting
cultures placed in 150 cc. sterile flasks, were added 15 cc. of toluene,
and after shaking, these solutions were analyzed for monosaccha-
rides by our modified Barfoed method. The residues in the
original flasks were boiled and analyzed in the same manner.
All six flasks were then incubated. After $27\frac{1}{2}$ and 99 hours the
incubated solutions were again examined for monosaccharides.

Fermentation of Alcohols.

Mannitol.— The medium used in this experiment consisted of a 3 per cent pure mannitol solution with the usual nutrient salts added in the correct proportions. Upon inoculation this medium was found to ferment vigorously with extremely active gas production and a rapid rise in acidity. This fermentation proved to be one of the abnormal type as shown by the maintained high acid value after the maximum was reached. The acidity data are plotted in Fig. 10. No data was obtained showing the extent to which this carbohydrate was fermented by the organism.

Glycerol.—A medium containing 3 per cent of pure glycerol was prepared and inoculated with active culture. After the carbohydrate contained in the added inoculum was consumed there was no evidence whatever of any fermentation of the glycerol. Hence it is concluded that this material is not available as a source of carbon for the butyl bacillus.

Hydrolysis of Carbohydrates in the Presence of Toluene.

Knowing from previous experiments that the organism is capable of utilizing such carbohydrates as sucrose, maltose, lactose, raffinose, melezitose, dextrin, starch, and inulin, as well as the simpler monosaccharides, the question at once arose as to the exact manner in which these compounds were attacked. In the case of colloidal substances such as starch, dextrin, and inulin, it is obvious that the organism must first effect hydrolysis before these materials can enter the cell. Hence it follows that the bacillus must secrete exocellular enzymes to effect these hydrolyses. Sugars, however, are capable of passing through the membrane with facility, and initial hydrolysis is not therefore essential. For each sugar there are three possible methods of attack; *viz.*, (1) direct utilization without preliminary hydrolysis; (2) preliminary hydrolysis by an exocellular enzyme; (3) hydrolysis within the cell by an enzyme not secreted. If the first of these methods obtains we must assume the presence within the cell of an elaborate enzyme system capable of converting a large number of sugars of very different structure into common end-products. On the other hand, it seems more probable that the di- and trisaccharides are first split into their respective simple hexoses either without

or within the cell, and that these sugars are then attacked by the acid-forming enzymes. The raffinose experiments have demonstrated in a definite manner the existence of sucrase. Assuming that lactase and maltase also exist, our conception of the enzyme system becomes much simpler. Any sugar, for instance, which, following hydrolysis, yields the hexoses, glucose, fructose, or mannose, can be fermented by the same enzyme system owing to the fact that these sugars are interchangeable, passing through a common enolic form when in solution. Galactose, however, when produced by hydrolysis, requires a special mode of attack, since this hexose does not pass into the same enolic form as do the other three sugars mentioned. Nevertheless, this special galacto-enzyme, which is required, exists in the butyl organism because we find that pure galactose is at least partially fermented and lactose completely.

Since there is little doubt but that the complex sugars are first hydrolyzed before they are fermented, the question still remains as to whether these hydrolyses take place outside or inside the cell. The problem is considered in the following series of experiments. The method of study, briefly stated, consisted of preparing flasks of the various carbohydrates; inoculating these with active cultures; incubating for about 24 hours; and finally adding toluene to stop further cell activity. Changes in the reducing sugar values were then noted and compared with suitable controls in order to determine whether any hydrolytic action had taken place by secreted enzymes.

Sucrose, Maltose, and *Lactose.*—To 300 cc. flasks, each containing 200 cc. of nutrient solution, were added 6 gm. of the sugar to be tested. The solutions were sterilized at 5 pounds pressure for $\frac{1}{2}$ hour. No hydrolysis occurred in the sucrose flask and only a slight amount in those containing maltose and lactose. Each flask was inoculated with 10 cc. of active culture and incubated at 37°C. for 26 hours. To 100 cc. portions of the actively fermenting cultures placed in 150 cc. sterile flasks, were added 15 cc. of toluene, and after shaking, these solutions were analyzed for monosaccharides by our modified Barfoed method. The residues in the original flasks were boiled and analyzed in the same manner. All six flasks were then incubated. After $27\frac{1}{2}$ and 99 hours the incubated solutions were again examined for monosaccharides.

This same experiment was repeated as a check on the results. The two sets of data are given in Table IX.

It is clearly shown that maltase is secreted by the butyl organism. On the other hand, there is no evidence of any secretion of sucrase or lactase. Therefore, maltose alone is hydrolyzed in part at least outside of the cell. This seems to be in keeping with our previous observation that in a mixture of glucose and maltose, the latter sugar is fermented simultaneously with the glucose. We find now that this is due to the immediate secretion of maltase which at once begins to attack the disaccharide present in the

TABLE IX.

Toluene Experiments.

After toluene addition.	Cuprous oxide per 25 cc. of solution.		
	Sucrose.	Maltose.	Lactose.
Series I.			
hrs.	*mg.*	*mg.*	*mg.*
0	0.0	5.1	0.5
27½	0 0	31 5	0.0
99	0.0	57.3	0.0
Series II.			
0	0.0	0 0	0.0
48	0 0	43 4	0.0
Boiled controls after 48 hrs......	0 0	Trace.	0.0

medium. In the case of mixtures containing either sucrose or lactose no suitable enzymes are secreted and we find that the disaccharides are not molested until all of the glucose has disappeared. What is the reason for this selection? Is it due to differences in the relative penetration of di- and monosaccharides through the cell membrane or is it due to a natural preference of the organism for the simpler and more easily oxidized food? At present we are unable to answer these questions.

Raffinose.—A raffinose medium, containing 3 per cent of the sugar, was inoculated with active culture and incubated for 20 hours after which toluene was added. No reducing sugars were

present. The flask was replaced in the incubator and allowed to remain for 72 hours. Upon examination with Fehling's solution no reducing sugar was found. This confirmed the previous observation that sucrase is not secreted.

Starch, Dextrin, and Inulin.—With regard to the polysaccharides, starch, dextrin, and inulin, experiments of a similar kind were performed. The changes taking place in the flasks to which toluene had been added and in the boiled controls were followed by means of sugar determinations and iodine color reactions. The results obtained show that in all three cases hydrolysis is effected by means of secreted enzymes. We conclude, therefore, that the two enzymes, amylase and inulinase, are secreted by the bacillus.

<div align="center">SUMMARY.</div>

1. The fermentations obtained with the various carbohydrates used in this investigation are of two types. The first type, to be regarded as the normal, is characterized by a decided fall in the acidity after the maximum is reached, and also by the complete consumption of the carbohydrate. The group of abnormal fermentations is characterized by the persistence of a high acidity and also by the incomplete destruction of the carbohydrate.

2. Glucose, fructose, mannose, sucrose, lactose, and starch belong to Group I, while galactose, xylose, arabinose, raffinose, melezitose, inulin, and mannitol constitute Group II. Dextrin belongs to either group depending upon the method used in the preparation of the sample fermented. The biological product is completely fermented, but the dextrin prepared by the acid hydrolysis of starch is only partially consumed.

3. Trehalose, rhamnose, melibiose, and glycerol are not fermented.

4. The butyl organism secretes the following enzymes: amylase, inulinase, and maltase; but it does not secrete sucrase, lactase, or raffinase.

5. Raffinose is hydrolyzed within the cell by sucrase into melibiose and fructose.

6. The organism first completely removes the hexoses, with the exception of galactose, from mixtures also containing sucrose and lactose. Maltose on the other hand is fermented concurrently with glucose, fructose, or mannose.

I wish to acknowledge with pleasure my indebtedness to Professor Horace B. Speakman for his ever ready counsel and advice throughout this work.

BIBLIOGRAPHY.

1. Beijerinck, M. W., Verhandl. K. Akad. Wetenschappen te Amsterdam, 1893, Section 2, pt. 1.
2. Perdrix, L., *Ann. Inst. Pasteur.*, 1891, v, 287.
3. Grimbert, L., *Ann. Inst. Pasteur.*, 1893, vii, 353.
4. Fernbach, A., and Strange, E. H., United States Patent, Nos. 1044368, 1044446, 1044447, 1912.
5. Weizmann, C., British Patent, No. 4845, 1915.
6. Speakman, H. B., *J. Biol. Chem.*, 1920, xli, 319.
7. Speakman, H. B., *J. Biol. Chem.*, 1920, xliii, 401.
8. Reilly, J., Hickinbottom, W. J., Henley, F. R., and Thaysen, A. C., *Biochem. J.*, 1920, xiv, 229.
9. Brown, H. T., Morris, G. H., and Millar, J. H., *J. Chem. Soc.*, 1897, lxxi, 94.
10. Legrand, *Compt. Rend. Acad.*, 1921, clxxii, 602.
11. Peterson, W. H., and Fred, E. B., *J. Biol. Chem.*, 1920, xlii, 273.
12. Fischer, E., *Ber. chem. Ges.*, 1895, xxviii, 1429.
13. Bau, *Chem. Ztg.*, 1902, xxvi, 69.
14. Browne, C. A., A handbook of sugar analysis, London, 1912.
15. Effront, J., and Prescott, Enzymes and their applications, New York, 1904.

ANIMAL CALORIMETRY.

TWENTY-SECOND PAPER.

THE PRODUCTION OF FAT FROM PROTEIN.

By H. V. ATKINSON, DAVID RAPPORT, and GRAHAM LUSK.

WITH THE TECHNICAL ASSISTANCE OF G. F. SODERSTROM AND JAMES EVENDEN.

(From the Physiological Laboratory of Cornell University Medical College, New York City.)

(Received for publication, April 21, 1922.)

In the second paper of this series Williams, Riche, and Lusk (1) described experiments which showed the hourly metabolism of a dog after giving 1,200 gm. of meat. The dog weighed 13.5 kilos and was maintained by administering 1,200 and 700 gm. of meat on alternate days. When given the larger quantity of meat, which contained about 1,200 calories, the heat production was about 800 calories daily, the dog resting quietly. The protein content of the meat was the equivalent of 900 calories. It follows that the protein ingested was alone more than sufficient for the dog's needs. However, when 700 gm. of meat were given the heat production of the resting quiet dog was about 700 calories daily, the ingested meat contained the same number of calories, while the protein element contained only 525 calories. Under these latter conditions one would not expect any permanent retention of glycogen derived from the protein metabolism of this dog during a day of ordinary activity following the administration of 700 gm. of meat.

The conditions were therefore favorable for the deposit of glycogen in the liver following the administration of 1,200 gm. of meat. As a matter of fact, during a period of 14 hours after the administration of this large amount of meat there was much less carbon eliminated in the respiration than corresponded to the protein metabolism of the time as measured by the excretion of

155

nitrogen in the urine. That this carbon was retained in the form of glycogen was proved by the fact that the quantity of oxygen absorbed agreed with this hypothesis, and that the heat calculated on the basis of a retention of glycogen agreed with the heat as measured by the calorimeter.

The Work of Atkinson and Lusk.

Fat Formation.—Atkinson and Lusk (2) performed a long series of experiments with the object of discovering the quality of the material retained when, after giving meat in large quantity to a dog, the amount of protein metabolism was in excess of the fuel needed for the heat production of the time.

A résumé of these experiments is given in Table I.

It will be remembered that when the protein of meat is oxidized in the body the respiratory quotient is 0.801.

It is evident from the table that in ten out of twelve experiments, after giving meat amounting to between 700 and 1,300 gm. daily, the respiratory quotients varied between 0.787 and 0.808, as appears below:

Experiment No..	31	48	54	51	47	46	55	30	34	32
R. Q.	0.787	0.793	0.794	0.795	0.795	0.796	0.797	0.800	0.800	0.808
R. Q. of deposit..	0.960	0.860	0.860		0.840			0.830	0.830	0.830	0.770

In Experiments 51 and 46 there was no retention of protein carbon, whereas in six of the experiments the retained carbon was held in such a form that, had it been oxidized, it would have yielded respiratory quotients of between 0.83 and 0.86, which indicates the retention of a pabulum containing only about half of its calories in fat and half in carbohydrate. By weight this would indicate the retention of approximately 1 gm. of fat to every 2 gm. of glycogen.

Only after the ingestion of meat in very large quantities— 1,100 and 1,300 gm.—was there evidence of the conversion of protein into fat as the dominant feature of the process. This appears below:

Experiment No.	33a	33b	56
R. Q.	0.831	0.843	0.826
R. Q. of deposit	0.680	0.490	0.710

As the respiratory quotient of fat is 0.707, the above results warranted the conclusion that in the case of excessive ingestion of meat by a dog the retained pabulum might be laid down as fat when the circumstances were favorable. It was noted that it was very difficult to induce the dog to take meat in these very large quantities.

During the period of experimentation twelve alcohol checks were made. The average of all the respiratory quotients was 0.668 (theory 0.667) and the heat recovered was 0.3 per cent greater then the heat calculated to be obtainable from the combustion of the alcohol.

The extent of the hourly retention of carbon calculated from the protein metabolism (measured by the urinary nitrogen) and the CO_2 output of the period bear no relation to the total heat production, as appears in the following tabulation:

Experiment No..	55	47	34	30	48	54	56	33a	31	32
Protein C retained, gm......	0.91	0.77	0.70	0.66	0.61	0.59	0.59	0.56	0.48	0.32
Calories of metabolism.......	32.00	29.90	34.00	31.50	29.40	31.10	31.60	31.70	34.30	34.90

It is well known that the deposition of glycogen does not increase the heat production. In Experiments 33a and 56, in which fat appeared to be the sole material deposited, the heat production was no higher than in Experiments 55, 47, 30, 48, 54, and 31, when fat and glycogen were laid down in about equal caloric equivalents.

It is therefore apparent that *the specific dynamic action of protein is not due to the formation or deposition of synthetically formed glycogen or fat.*

Basal Metabolism and "Deposit Protein." —"Deposit protein" is that quantity of protein which is held in the body after excessive protein ingestion, and which is gradually eliminated from the cells in which it is stored upon the cessation of a high protein diet. Historically it is the same as Voit's "circulating protein." Benedict, Miles, Roth, and Smith (3) were the first to associate the loss of "deposit protein"—which they called "surplus cellular nitrogen"—with a reduction in the total metabolism of men.

TABLE I.

Effect of Meat Ingestion on Hourly Metabolism. Series I. Dog XVIII.

Experiment No.	Date.	Food.	Number of hours.	Urine N.	R. Q.	Calories. Indirect.	Calories. Direct.	C deposited.	R. Q. of deposited material.	Hours after food.
	1919			gm.				gm.		
27	Feb. 6	Basal.	2	0.15	0.810	15.92	16.08			(Weight = 11.24 kg.)
30	" 18	Meat, 800 gm.	3	1.46	0.800	31.47	32.75	+0.63	0.83	4, 5, 6
31	" 19	" 900 "	3	1.47	0.787	31.33	34.11	+0.48	0.96	4, 5, 6
32	" 20	" 1,000 "	4	1.46	0.808	34.90	35.87	+0.32	0.77	4, 5, 6, 7
33	" 21	" 1,100 "	2a	1.45	0.831	31.65	31.36	+0.56	0.68	4, 5
			2b	1.45	0.843	35.28	31.54	+0.25	0.49	6, 7
34	Feb. 22 / " 23 / " 24	Meat, 700 gm. / " 700 " / " 1,080 " *	2	1.57	0.800	34.00	34.12	+0.70	0.83	4, 5
35	" 26	Basal.	3	0.27	0.820	19.74	19.59			(Weight = 12.07 kg.)
36	" 27	"	3	0.20	0.830	18.25	17.16			
37	" 28	"	2	0.17	0.850	17.30	16.95			
38	Mar. 1	"	2	0.15	0.820	18.21	(18.21)			
39	" 3	"	3	0.15	0.850	17.57	17.22			
43	" 12	"	2	0.15	0.810	17.08	16.99			(Weight = 11.50 kg.)

	Date	Diet								Remarks
46	Mar. 17	Meat, 1,200 gm.	3	1.02	0.796	26.57	28.10	0	0.81	5, 6, 7 after 1 day's fast.
47	" 18	" 800 "	3	1.44	0.795	29.90	30.77	+0.77	0.86	5, 6, 7
48	" 19	" 800 "	4	1.35	0.793	29.37	30.27	+0.61		5 to 8
49	" 22	Basal.	2	0.23	0.790	17.72	17.54			
50	" 24	"	2	0.16	0.840	17.26	16.87			
51	" 28	Meat, 800 gm.	4	1.02	0.795	27.04	27.52	+0.59	0.86	5 to 8 after 4 days' fast.
54	Apr. 15	" 800 "	4	1.41	0.794	31.07	30.57	+0.91	0.83	5 to 8
55	" 16	" 1,000 "	1	1.58	0.797	31.97	31.98	+0.59	0.71	5 to 8
56	" 19	" 1,300 "	1	1.47	0.826	31.62	33.25			5 to 8 after 1 day's fast.
Total						568.22	571.85			

* Standard diet at 5 p.m. and thereafter daily until Mar. 15.

TABLE—III *The Effect of Meat Ing*

Date	Experiment No.	Time	CO_2	O_2	R.Q	H_2O	Urine N.	Non-protein CO_2	Non-protein O_2	Non-protein R.Q	Pro-tein	p...
1920			gm.	gm.		gm.	gm.	gm.	gm.			
Mar. 26	80	12.21— 1.28	6.70	7.34	0.664	4.84						
		1.28— 2.28	6.16	6.70	0.669	4.24						
					0.667							
Apr. 14	68	12.48— 1.18	4.93	4.17	0.860	7.48	0.103			0.875	2.73	
	Dog XVIII	1.18— 2.48	4.80	4.28	0.816	6.76	0.103			0.819	2.73	
Apr. 17	70	12.40— 1.40	10.10	5.14	0.842	11.83	1.440	−3.36	−3.45	0.708	38.17	—
	Dog	1.40— 2.40	10.45	5.32	0.845	11.94	1.440	−3.01	−3.18	0.688	38.17	—
	XVIII	2.40— 3.40	10.56	5.38	0.845	10.69	1.440	−2.90	−3.08	0.685	38.17	—
					0.844					0.694		
Apr. 19	81	3.04— 4.04	6.45	7.03	0.667	4.57						
		4.04— 4.44	4.46	4.80	0.676	3.06						
					0.670							
1922												
Mar. 6	141	1.41— 2.41	8.17	8.95	0.664	6.14						
		2.41— 3.41	8.09	8.93	0.659	5.87						
		3.41— 4.41	8.01	8.75	0.666	5.78						
					0.663							
Mar. 8	142	11.26—12.26	8.33	8.98	0.674	6.12						
		12.26— 1.26	8.27	9.21	0.653	5.94						
					0.664							
Mar. 8	75	2.50— 3.50	11.54	10.36	0.810	13.86	1.80	−5.32	−4.88	0.786	47.80	—
	Dog XIX	3.50— 4.50	11.73	9.97	0.856	13.19	1.80	−5.13	−5.27	0.708	47.80	—
					0.833					0.749		
Mar. 11	144	10.21—11.21	7.75	8.53	0.664	5.97						
		11.21—12.21	7.66	8.41	0.662	5.65						
					0.662							

* Calorimeter cold.

Hourly Metabolism.   Series II.

	Direct.	Body temperature Start.	End.	Morning weight.	Behavior of dog.	Food.
				kg.		
1	24.50					
4	21.95					
5	46.45					
4	12.76	37.81		8.57	Quiet.	Basal metabolism.
5	14.31		37.45		"	
9	27.07					
5	27.52				Quiet.	1,000 gm. of meat at 8.33 a.m. 3rd day of same
4	29.07				"	diet. Also 100 gm. of biscuit meal at 5 p.m. for
7	28.73		38.48		"	previous 2 days.
6	85.32					
6	23.47					
1	16.12					
7	39.59					
6	30.24					
7	29.76					
8	29.95					
1	89.95					
5	32.66					
3	31.75					
8	64.41					
4	30.89	38.72			Quiet.	1,000 gm. of meat at 9 a.m. 3rd day of same diet.
9	33.42		38.65		"	Standard diet also given constantly at 5 p.m.
4	64.31					
4	26.65*					
7	27.78					
	54.43					

Animal Calorimetry

Date.	Experiment No	Time.	CO_2	O_2	R.Q	H_2O	Urine N.	Non-protein.			Pro-tein.	N pro
								CO_2	O_2	R.Q.		
			gm.	gm.		gm.	gm.	gm.	gm.			
1923 Mar. 11	77	1.54— 2.54	12.85	10.92	0.856	22.65	1.803	−4.01	−4.32	0.675		
		2.54— 3.54	13.40	11.65	0.837	19.85	1.803	−3.46	−3.59	0.701		
					0.846					0.687		
Mar. 15	145	11.34-12.34	7.03	8.30	0.669	5.67						
		12.34- 1.34	7.31	8.16	0.652	5.42						
					0.660							

In Table I it will be noted that the basal metabolism of a dog maintained upon the ordinary "standard diet" (meat, 100 gm.; biscuit meal, 100 gm.; lard, 20 to 30 gm.) was 16 calories per hour. After 8 days of meat ingestion as the sole diet, administration of the "standard diet" was resumed. 18 hours after the second administration of the "standard diet" the basal metabolism was 19.7 calories and then on successive days was measured as·being 18.3, 17.3, 18.2, and 17.6 calories. Even after $2\frac{1}{2}$ weeks the basal metabolism was 17.08 calories, showing a persistently higher level than had obtained before the ingestion of meat.

The bodily condition is always a factor to be considered in the determination of basal metabolism. The condition of muscular strength, accompanied by the addition of an "improvement quota" of protein (4) to the cells of the body, results also in a higher metabolism in the dog (5) and in man (6). It is possible that a lesser amount of "deposit protein" and of "improvement protein" may be in part responsible for the lower basal metabolism of women, first pointed out by Gephart and Du Bois (7). Thus the recent experiments of Blunt and Bauer (8) show that under-nutrition does not play a large part in reducing the metabolism of women.

The Experiments of Rapport and Lusk.

The problem of the production of fat from protein seemed of sufficient significance to endeavor to follow the same with the

ncluded.

		Body temperature		Morn-ing weight.	Behavior of dog.	Food.
di-t.	Direct.	Start.	End.			
				kg.		
63	36.47	39.04		13.0	Quiet.	Meat 1,000 gm. at 9 a.m. 6th day of same diet at
02	36.44		38.76		"	9 a.m., with standard diet also daily at 5 p.m.
65	72.91					
23	26.93					
06	26.65					
29	53.58					

greatest care as to the accuracy of the determination of the individual respiratory quotients after meat ingestion.

An additional procedure was added in this series. The standard diet, containing 70 gm. of starch, was given every evening at 5 p.m. in order to charge the glycogen reservoirs of the body. The meat was given early in the morning. The complete data are to be found in Table II. The accuracy of the experiments may be gauged from the following figures:

Date.		R. Q.	R. Q. of retained material
1920			
Mar. 26	Alcohol check.	0 667	
Apr. 17	Meat ingestion.	0.844	0 694
" 19	Alcohol check.	0.670	
1922			
Mar. 6	" "	0 663	
" 8			
a.m.	" "	0 664	
p.m.	Meat ingestion.	0.833	0 749
Mar. 11			
a.m.	Alcohol check.	0 662	
p.m.	Meat ingestion.	0.846	0.687
Mar. 15	Alcohol check.	0 660	

If protein is converted into fat two conditions must be ful-filled: (*a*) the R. Q. must be higher than that of protein itself

and (b) there must be a retention of protein carbon. The urinary nitrogen is usually higher in the dog when the animal is catheterized hourly (1) than it is when it accumulates in the bladder during a calorimeter period, and the true index of protein metabolism is accounted to be the level to which the urinary nitrogen rises when the urine is fractionated hourly on a day that the dog is given the same diet but is not in the calorimeter.

The method of calculation of the 5th hour after giving 1,000 gm. of meat to Dog XVIII will suffice as a guide to all the experiments.

Experiment 68.

Urinary N = 1.44 gm.

	CO_2 gm.	O_2 gm.	Calories.
Equivalent of 1.44 gm. of N.................	13.46	12.17	38.17
Found in respiration........................	10.10	8.72	
	3.36	3.45	11.32

Calories indirect...................................... 26.85

R. Q. of deposit = 0.708
Value of fat deposited = 11.32 calories
Calories (indirect) = 26.85
Calories (direct) = 27.52
C retained = 0.92 gm. $\begin{cases} = 1.2 \text{ gm. of fat} \\ = 2.3 \text{ gm. of glucose (8.63 calories)} \end{cases}$
Calories if C had been retained as glycogen = 29.54

The respiratory quotients and the heat directly measured confirm the conception that fat and not glycogen was the form in which the carbon was deposited.

If one makes use of this method of calculation for the average hourly heat production for the series of three experiments, one may construct Table III.

It is evident that, computed on the oxygen absorption by the method of indirect calorimetry already outlined, the calculated heat production is almost exactly the same as it is when the computation is based upon the hypothesis that the carbon retained is laid down in the form of fat (1 gm. of C retained as fat = 12.31 calories).

The agreement between direct and indirect calorimetry is not as close as one would wish, though in this regard the alcohol checks agree on the average within 1 per cent. The calories as calculated are 3.8 per cent less than the calories found. However, if the carbon retention is assumed to take the form of glycogen, then the calculated heat would be 6.3 per cent higher than the amount directly measured. The findings, therefore, favor the idea of the retention of a pabulum in the form of fat.

TABLE III.

Direct and Indirect Calorimetry in Hourly Periods after the Ingestion of 1,000 Gm. of Meat.

	Experi-ment No	Urine N	Protein C to body.	Calories.			
				Direct.	Indirect.	C deposited as fat.	C deposited as carbo-hydrate.
		gm.					
Dog XVIII..	68	1 44	0 84	28 44	27 55	27 83	30 30
Dog XIX....	75	1 80	1 40	32 16	30 97	30 51	34 57
	77	1 80	1 02	36 46	34 83	35.25	38 29
Total........				97 06	93 35	93 59	103 16

Here, as in the first series of experiments, the height of the metabolism is not proportional to the quantity of protein carbon retained. In Experiment 77, high humidity in the calorimeter probably produced increased respiratory activity and in consequence increased metabolism. The older literature has been elsewhere reviewed (9), but these experiments, by a new method, add another link to the chain of evidence that protein may be converted into fat. It must be remembered, however, that the conditions were exceptional, in that the animals were maintained upon a nutritive plane which would fill the glycogen reservoirs, were given meat up to the limit of their willingness to consume it, and were kept in absolute rest in a calorimeter at an environmental temperature of 25°C. Under these circumstances fragments of protein metabolism, which would ordinarily have been oxidized or converted into glucose and laid down as glycogen, found no other pathway open than conversion into fat. Under a natural diet these conditions would not exist.

SUMMARY.

1. When the glycogen reservoirs of the body are low the ingestion of meat in large quantity results in the deposition of glycogen.

2. The continued ingestion of much meat brings about the retention in the body of a pabulum consisting partly of glycogen and partly of fat. Only when meat in very great excess is given is fat alone retained.

3. When a carbohydrate-containing meal is given in the evening and 1,000 gm. of meat in the morning, then during the height of protein digestion the respiratory quotient indicates a production of fat from protein.

4. Following the prolonged ingestion of meat in large amounts, which induces the retention of "deposit protein," the basal metabolism may rise from a former level of 16 calories per hour to one of 19.7, an increase of 23 per cent, from which level it slowly falls with the gradual elimination of "deposit protein."

BIBLIOGRAPHY.

1. Williams, H. B., Riche, J. A., and Lusk, G., *J. Biol. Chem.*, 1912, xii, 349.
2. Atkinson, H. V., and Lusk, G., *Proc. Nat. Acad. Sc.*, 1919, v, 247.
3. Benedict, F. G., Miles, W. R., Roth, P., and Smith, H. M., *Carnegie Inst. Washington*, *Pub. 280*, 1919.
4. Rubner, M., *Arch. Physiol.*, 1911, 76.
5. Lusk, G., *J. Biol. Chem.*, 1915, xx, 555.
6. Benedict, F. G., and Smith, H. M., *J. Biol. Chem.*, 1915, xx, 243.
7. Gephart, F. C., and Du Bois, E. F., *Arch. Int. Med.*, 1916, xvii, 902.
8. Blunt, K., and Bauer, V., *J. Home Econom.*, 1922, xiv, 171.
9. Lusk, G., The elements of the science of nutrition, Philadelphia and London, 3rd edition, 1917, 228–30.

THE LIPOIDS OF THE BLOOD IN TUBERCULOSIS.

By B. H. HENNING.

(From the Department of Biochemistry and Pharmacology, University of California, Berkeley.)

The investigation of fat metabolism in the body has involved the study of the fats and other lipoids as they occur in the intestine, the blood, and the tissues. In the blood (1) the lipoids are found normally in a fairly constant quantitative relationship to each other. The work of Brinkman and van Dam (2) has recently emphasized the physiological antagonism between lecithin and cholesterol, which was found by earlier workers (3). Bloor (3) and others have shown that whenever one of the blood lipoids is high, similarly high values are to be expected for the others; and especially as regards lecithin and cholesterol is the balance carefully preserved. An extension of this study to diseases of a metabolic nature has led Rudolf (4) to consider the lipoid picture as of considerable diagnostic value, because he found that in each disease he studied there was a definite change from the normal. Special studies have been made of the blood lipoids in diabetes (5), nephritis (6), and the anaemias (7). Nowhere has any attention been paid to the condition of the blood lipoids in tuberculosis, although certain phases of this disease would lead one to expect some abnormality of fat metabolism. Thus there is a toxemia with resulting emaciation, formation of the characteristic tubercles which contain much lipoid material, and the use in treatment of a fat-rich diet. It seemed desirable, therefore, to extend the study of the blood lipoids to tuberculosis and in the following paper are reported observations made on a series of twenty-one cases of advanced tuberculosis, mainly pulmonary.

Subjects.—All the subjects were patients in the tuberculosis wards of the San Francisco Hospital. All had chronic pulmonary

tuberculosis, advanced, giving negative Wassermann reaction and were apparently free from intercurrent infections. Blood from Cases 1 to 9 inclusive was drawn October 22, 1921, and from the remainder December 15. 1921. Cases 2 and 11 are the same man. His blood was used a second time to check the method.

TABLE 1.

Lipoids in the Blood in Tuberculosis.

| Cases. | Cholesterol. | | Lecithin. | Fatty acids. | | | | Lecithin / Cholesterol | Cholesterol / Fatty acid |
	Saponi-fication	Non-saponi-fication.		Total.	Fatty acid in lecithin.	Fatty acid in choles-terol esters.	Resid-ual fatty acid.		
1	116	202	203	380	134	67	179	1.00	0.53
2	82	190	216	362	144	63	155	1.13	0.52
3	126	168	203	336	134	56	146	1.20	0.50
4	104	244	240	398	160	81	157	0.99	0.61
5	138	236	227	362	152	78	132	0.96	0.65
6	106	202	221	380	146	67	167	1.09	0.53
7	136	250	245	392	162	83	147	0.98	0.63
8	198	214	226	396	150	71	175	1.05	0.54
9	112	174	206	348	136	58	154	1.18	0.50
10	86	169	227	346	150	56	140	1.34	0.46
11	91	228	240	375	160	76	139	1.05	0.60
12	90	219	214	362	142	73	147	0.97	0.60
13	91	201	229	344	152	67	125	1.13	0.58
14	108	206	207	372	138	68	166	1.00	0.55
15	76	174	202	339	134	58	147	1.17	0.51
16	82	186	205	369	136	62	171	1.10	0.50
17	90	196	240	378	160	65	153	1.22	0.51
18	90	232	201	361	148	77	136	0.87	0.64
19	97	214	217	375	144	71	160	1.01	0.57
20	96	198	214	363	142	66	155	1.08	0.54
21	99	180	192	334	128	60	146	1.06	0.53

Cases 7 and 8 were discharged from the Hospital as improved. Case 21 was very weak and emaciated, the lowest results were obtained from him.

Methods.—The methods which were used for this work for the quantitative determination of the blood lipoids are those described in the various contributions of Bloor (8, 9, 10). Determinations were made only on the plasma since the experience of workers in this field has shown that, except in alimentary lipemia, the lipoid

composition of the corpuscles is relatively constant. Cholesterol was determined on the blood extract both with and without saponification since it was found that values by the two methods were often strikingly different.

Calculations.—Values for cholesterol, fatty acid, and lecithin were obtained directly while the remaining values were calculated from these. Fatty acid in cholesterol esters was calculated to be one-third of the total value of cholesterol determined by the non-saponification method, while that in lecithin was taken as two-thirds. The sum of these two values subtracted from the value for total fatty acids gives the amount of fatty acid in combinations other than those mentioned. The ratios, $\frac{\text{lecithin}}{\text{cholesterol}}$ and $\frac{\text{cholesterol}}{\text{fatty acid}}$, were calculated only for the non-saponification values of cholesterol. Values expressed in milligrams per 100 cc. of plasma are contained in Table I.

DISCUSSION.

Cholesterol.—Cholesterol was found by the non-saponification method to be practically normal, but the saponification method gave results uniformly much lower (averaging about 50 per cent) except in Case 8, and it is possibly significant that in this case improvement had occurred and the patient was allowed to leave the hospital. The difference between the values for cholesterol by the two methods is very striking and since ordinary cholesterol is not appreciably affected by saponification the presence of some substance other than cholesterol which gives the cholesterol color reaction but which is sensitive to alkali is indicated. Since the value found without saponification is the same as similar values for normal blood it appears that in tuberculosis true cholesterol is replaced in considerable part by the unknown substance, which may, however, be closely related to cholesterol since it gives the same color reaction. The reason for the apparent substitution for cholesterol in tuberculosis blood is at present a matter of speculation, but when the lipoid nature of the tubercle bacillus and of the tubercles is considered, it seems reasonable to suspect that cholesterol may be involved in some way in the attempt of the body to protect itself from this organism.

Lecithin and Fatty Acid.—These substances were present in amounts comparable with values for the normal published by Bloor. The residual fatty acid is relatively high and since there was no visible lipemia the fatty acids could hardly be present as fat, so that some other form of combination is suggested. The ratios, $\dfrac{\text{lecithin}}{\text{cholesterol}}$ and $\dfrac{\text{cholesterol}}{\text{fatty acid}}$, are fairly constant for the entire series. They do not vary widely from those given for normal individuals.

CONCLUSIONS.

1. Cholesterol was found to be uniformly low in tuberculous blood when determined by the saponification method, but normal when determined without saponification. The presence in the blood plasma in tuberculosis of a relatively large amount of an unknown substance which is probably closely related to cholesterol is thus indicated.

2. Total fatty acid and lecithin were within the normal range of values for these substances.

3. The "residual" fatty acid of the blood was high and since there was no lipemia the presence of other forms of fatty acid combination than those ordinarily present is suggested.

BIBLIOGRAPHY.

1. Bloor, W. R., *Physiol. Rev.*, 1922, ii, 92.
2. Brinkman, R., and van Dam, E., *Biochem. Z.*, 1920, cviii, 35.
3. Bloor, W. R., *J. Biol. Chem.*, 1914, xix, 1.
4. Rudolf, J., *Z. physiol. Chem.*, 1917–18, ci, 99.
5. Bloor, W. R., *J. Biol. Chem.*, 1916, xxvi, 417.
6. Bloor, W. R., *J. Biol. Chem.*, 1917, xxxi, 575. Bloor, W. R., and MacPherson, D. J., *J. Biol. Chem.*, 1917, xxxi, 79.
7. Bloor, W. R., *J. Biol. Chem.*, 1916, xxv, 577.
8. Bloor, W. R., *J. Biol. Chem.*, 1914, xvii, 377.
9. Bloor, W. R., *J. Biol. Chem.*, 1916, xxiv, 227.
10. Bloor, W. R., *J. Biol. Chem.*, 1915, xxii, 133.

FAT EXCRETION.

By ELSIE HILL and W. R. BLOOR.

(From the Department of Biochemistry and Pharmacology, University of California, Berkeley.)

The feces of animals normally contain some fatty material which consists mainly of fatty acids and their salts (soaps), a smaller amount of cholesterol and its derivatives, and a little fat. The fatty acids and fats are generally assumed to be unabsorbed residues of the fat of the food and a food fat is said to be well or poorly absorbed according as the amount of fatty substance recoverable from the feces is relatively small or large. In some cases, and especially when the amount is large, there is little doubt that the assumption is approximately correct and that the fat and fatty acids found represent largely unabsorbed food fat, but there is considerable evidence to indicate that in many or perhaps most instances the feces fat has no direct relationship to the fat of the food, but represents rather some form of excretion from the intestinal tract. Friedrich Müller (1) in studies of the feces fat of the two professional fasters Cetti and Breithaupt found in the case of Cetti that the fatty material of the fasting feces was about 36 per cent of the dry material and that 39 per cent of it was fat, 45 per cent fatty acids, and 16 per cent cholesterol. In the case or Breithaupt the fatty material composed 28 per cent of the dry matter and of this, neutral fat and cholesterol formed 47 per cent, fatty acids (and soaps) 53 per cent. In two periods with food on the same subject the lipoid material formed, in the first period, 24 per cent of the dry material, and of this 36.5 per cent consisted of neutral fat and cholesterol and 62.6 per cent of fatty acid. In the second food period the lipoid material constituted 28 per cent of the dry substance, and of this 29 per cent was neutral fat and 71 per cent fatty acid and

soap. The percentage of total lipoid material in the feces during the food period thus differed but little from that of the fasting period, although the proportion of fatty acid and soap was relatively considerably higher during the food period. Also, since the amount of feces was greater, the absolute amount of lipoid material excreted during the feeding period was greater than during the fasting period. In the same article Müller refers to some earlier experiments (2) with dogs in which he found a lipoid content in the feces of 20 to 47 per cent of the dry substance, consisting mainly of fatty acid. Thus in one dog (2) weighing 23 kilos. in a 7 day fast the excretion was about 2.68 gm. of dry material per day, of which the fatty material amounted to about 34 per cent, and consisted of 67 per cent of fatty acid (and soap) and the remainder of neutral fat and cholesterol. He believed that this material originated as an excretion of the intestine and pancreas. In another dog weighing 18 kilos, on a diet of lean meat the feces amounted to 6.1 gm. of dry matter per day and contained 25 per cent of fatty material, of which 62 per cent was fatty acid and 38 per cent cholesterol, fat, etc. The addition of small amounts of fat to the diet affected the lipoid content of the feces only slightly, but larger amounts increased the lipoid output. The latter finding is not, however, incompatible with his assumption of a fat excretion, since where large amounts of fat are ingested a larger excretion might be expected.

Hermann (3) isolated loops of intestine and found that they filled up in the course of 3 or 4 weeks with material very similar to feces, and an examination of this material by Ehrenthal (4) demonstrated the presence of fat, soaps, and cholesterol. F. Voit (5) repeated the work and confirmed their results, finding that the contents of the intestinal ring had the same composition as hunger feces and almost the same as meat feces. In addition to ash and nitrogen there was always fatty material to the extent of 30 to 36 per cent, of which up to one-third (generally less than one-tenth) was neutral fat, one-half to four-fifths free fatty acid, and one-tenth to one-third soaps.

By the use of fistulas further information regarding intestinal secretion was obtained. Gumilewski (6) obtained from a low Thiry-Vella fistula in dogs a continuous secretion which was small in amount in fasting, 1 cc. per hour from an 11 cm. length of

intestine, but increased to 7 to 10 cc. during digestion. Röhmann (7) found little or no secretion from a high loop while considerable secretion was obtained from a low loop. Some time ago one of us (B) examined the secretion from a permanent Thiry fistula (consisting of about 14 inches of jejunum) in a healthy dog.[1] The secretion was collected on fat-free pads of gauze which were then boiled out with alcohol to extract the lipoid material. The alcoholic extracts were evaporated to small volume, diluted with water, acidified, the fatty matter was extracted with ether, the solvent evaporated, and the residue dried and weighed. In a 5 day period a total of 0.72 gm. of lipoid material consisting almost entirely of fatty acids was recovered. The animal was well fed during the period. It was not possible to repeat this important experiment at the time and the dog was disposed of. Numerous attempts have been made since then to obtain suitably operated animals but none survived the operation and resulting complications long enough to be used for an experiment.

The results noted above indicate that much fatty material is to be found in the intestine and feces which are entirely independent of the food. The question as to whether it is to be regarded as a secretion is complicated by the fact that in the feces and in intestinal loop contents there is much cellular material (bacteria, etc.), which contains fatty compounds and contributes to the "fat" content. The importance of this source of fat cannot be estimated. As regards the contents of intestinal rings, Voit (5) does not regard it as important since the nitrogen content is too low and the ash too high to be of cellular origin. The fact that the free flowing secretion from the intestinal fistulas contains little cellular material but considerable soap points to a true secretion.

Aside from the undetermined influence of lipoid from cellular material it is reasonable to assume that if the fatty material of the feces represents a true excretion of the intestine its nature would be independent of the food and of the food fat. If it represents unabsorbed food residues its nature would depend on that of the food fat. Certain modifications of this general assumption should probably be made in view of the probability

[1] Obtained through the kindness of Dr. Barney Brooks of the Washington University School of Medicine, St. Louis.

that on the one hand some of the food fat after absorption may be excreted into the intestine and on the other that the absorption of food fat from the intestine may be selective, less desirable, and possibly similar portions of all types of fat being rejected and appearing in the feces.

With these ideas in mind feeding experiments were carried out with two fats of widely different composition, and the feces fat was examined. Cats were used for the experiments. They were kept in cages throughout the period but allowed the free run of the room during a portion of the day for exercise. Their basal diet was a practically fat-free mixture of starch and extracted casein with meat extract for flavoring and bone ash to provide bulk. Experiments were conducted with (a) a diet of lean meat, (b) the basal diet alone, (c) the basal diet plus olive oil, (d) the basal diet plus coconut oil; in every case making the amount of the daily food such as to supply 100 calories per kilo of body weight. Generally the food was entirely eaten up, but occasionally when olive oil was fed and more frequently with coconut oil it was necessary to feed forcibly a portion of the oil in order to make sure that the cats received the required amount. Although apparently remaining in good health the animals lost weight steadily throughout the experiment.

The meat used contained from 2 to 7 per cent of fat with an iodine number of about 46 and a melting point of about 43°C. The casein was mainly prepared fresh from skim milk, but some commercial casein was used. In preparing it for use all samples were extracted with alcohol and ether and as used the casein-starch basal diet contained only a few milligrams of fatty substance in 100 gm. The coconut oil was fed in the form of the commercial butter substitute "Nucoa." This substance consists almost entirely of coconut oil but the flavor is somewhat disguised and is less objectionable than that of the commercial oil. The fatty acids of the material used melted at about 25°C. and had an iodine number of 8.8. The olive oil had an iodine number of 88.2.

Each experiment lasted a week, the periods being marked off by charcoal. The feces were collected as passed, kept in a stoppered bottle under 95 per cent alcohol until the end of the period when the whole was transferred to a large Erlenmeyer

TABLE I.

Feces "Fat" on Various Diets.

Subject No.	Fat eaten.	"Fat" in feces	Percentage of "fat" in feces to fat in diet.	Iodine No. of feces "fat."	M.P of feces fat
	gm.	*gm.*	*per cent*		°C.
Fat-free diet.					
1		1.70		36.3	3S
		3.29		32.6	42
2		0 86		37.1	26
3		1.47		27.4	39
4		1.47		30.0	30
Meat diet.					
1	50	6 00	12.0	39.0	44
	2S	0 S7	3 1	43 S	34
2	34	2 37	6.9	34 8	33
3	2S	1 10	3 9	42 3	36
4	2S	3.S4	13 7	30.5	35
Coconut oil.					
1	44	2 17	5.1	25 0	32
2	45	2.46	5.5	24.4	34
3	24.3	1.S2	7.6	31 0	30
4	52	3.55	6.9	21.6	27
Olive oil.					
1	30	1.50	5 0	3S.7	30
	50	2.00	4 0	53.2	26
2	45	2.51	5 6	41.0	32
	50	1.S1	3 6	44.S	30
3	50	2.76	5.5	49.S	33
	50	1.33	2.7	52.2	25
4	50	3 0	6.0	44 3	37
	50	3 0	6.0	32.7	40
Averages.					
Fat-free diet......		1 76		32.7	35
Meat diet		2.S3	7 9	3S 1	36
Coconut oil.......		2 50	6 3	24 S	31
Olive oil..........		2.24	4 S	44 6	31

flask. Alcohol was added to cover the material, then 10 gm. of stick potassium hydroxide, after which the flask was connected with a reflux condenser and boiled for 5 hours. At the end of the time the mixture was diluted with water, acidified with hydrochloric acid, and completely extracted with ether. The extracts were united, washed with water, the ether was distilled off, and the residue dried in a vacuum desiccator for 24 hours. The residue was then extracted with petroleum ether, the extract filtered, the ether distilled off, and the fatty residue dried as before and weighed. In this series of experiments no account was taken of the cholesterol-like substances (unsaponifiable matter). In determining their fat content the food materials were treated in the same way as the feces. Iodine number determinations were made by the Wijs method. Five experiments were carried out with the fat-free and meat diets, four with coconut oil, and eight with olive oil. The results of the experiments are given in Table I.

<div align="center">DISCUSSION.</div>

Total Fat.—The total "fat" in the feces varies a great deal on all the diets so that the average has not much meaning; but inspection of the data on the experiments will show that, as would be expected, there is less fat in the feces on the fat-free diet than on the others. Of the other three the meat diet yields the largest amount, due probably to enclosure of fat by the tissue, and the olive oil the least. Probably for the same reason (enclosure) the percentage feces fat of food fat is highest on the meat diet.

Iodine Number.—The iodine number is highest on the olive oil diet and lowest on the coconut oil, but in neither case does it approach the values of the fat fed, having rather a value not so greatly different from that of the fat-free diet. It is plain that the fat of the food has some influence on the feces fat but the influence is not great, especially when the amounts of fat fed are moderate, as in these experiments.

Melting Point.—The melting point of the feces fat is relatively constant, but is consistently lower on the fat diets than on either the fat-free or the meat diet, showing again the influence of the fat of the diet on the feces fat. The melting point of the feces fat is almost always below body temperature.

These results show. in agreement with the work of earlier investigators, that "fat" is to be found in the feces whether it is present in the food or not, and that fat in the food increases the feces fat, but only to a comparatively small extent. They show further that the nature of the "fat" of the feces is to a great extent independent of the food fat, being much the same no matter which of two widely different fats were fed or whether none was fed at all. Taken altogether they indicate a continuous output of "fat" in the feces of a constant composition independent of the diet.

The constancy of the feces fat independent of the diet favors the idea of a fat excretion, but might, of course, be explained as due to a constant output of cellular waste from the intestine and from bodies of bacteria. The answer of Voit to this possibility (see above) is not convincing, since the presence of much cellular material in the feces cannot be denied. The fact that fatty material can be collected from isolated portions of the intestine under conditions which to a large extent exclude a cellular origin (Thiry fistula) is a better answer to the objection, but unfortunately there is not much evidence of this nature available. It is not unlikely that feces fat may have more than one origin just as the fatty material from the skin comes partly from desquamated epithelium, and partly from the sebaceous glands.

SUMMARY.

When moderate amounts of fat are fed the fat of the feces is largely independent of the diet, and in composition approaches that from a fat-free diet.

The comparative constancy of composition of the feces fat favors the idea of a fat excretion from the intestine but while an excretion is probable it cannot be regarded as proven in view of the undetermined influence of lipoid from free cellular material.

The feces fat cannot ordinarily be regarded as unabsorbed food fat and, therefore, feeding experiments as a test of the extent of utilization of food fat are of doubtful value unless account be taken of the amount and kind of fat which appears in the feces independently of the food.

Fat Excretion

BIBLIOGRAPHY.

1. Müller, F., *Virchows Arch. path. Anat.*, 1893, cxxxi, 106.
2. Müller, F., *Z. Biol.*, 1884, xx, 327.
3. Hermann, L., *Arch. Physiol.*, 1890, xlvi, 93.
4. Ehrenthal, W., *Arch. Physiol.*, 1891, xlviii, 75.
5. Voit, F., *Z. Biol*, 1892, xxix, 325.
6. Gumilewski, *Arch. Physiol.*, 1886, xxxix, 556.
7. Röhmann, F., *Arch. Physiol.*, 1887, xli, 411.

ON THE COLORIMETRIC DETERMINATION OF HEMO-GLOBIN WITH ESPECIAL REFERENCE TO THE PRODUCTION OF STABLE STANDARDS.

By EDWIN H. TERRILL.

(From the Metabolic Unit, Department of Medicine, Washington University School of Medicine, St. Louis.)

CONTENTS.

INTRODUCTION.

Sahli demonstrated as the underlying principle of his well known hemometer, that the color intensity of an acid hematin solution varies directly as the amount present, a relation which does not, however, hold for all colored compounds. This allows the preparation of solutions of any desired strength from more concentrated ones according to the simple rules employed in volumetric work. But owing to a certain lack of permanence of all dilute acid hematin solutions, new ones must be prepared at intervals depending to some extent upon the method of preparation and preservation. These are best standardized by comparison with blood whose hemoglobin has been determined by the oxygen capacity method of Van Slyke, as recently modified by Van Slyke

and Stadie (1). The latter method, although very accurate, requires considerable experience in order to obtain consistent results. Attempts have therefore been made to devise a standard which limits as far as possible both the number and frequency of such checks.

Cohen and Smith (2), and later Robscheit (3) employed concentrated stock solutions of acid hematin from which dilutions were prepared at frequent intervals.

The method as described by these authors has several objectionable features. On standing, the stock solution deposits a sediment, and dilutions are always very turbid, more so when prepared from old solutions; also changes in color can be demonstrated often within 2 months, sufficient to prevent accurate color matching. Fading occurs to a variable degree, although the turbidity and color change after a time make it difficult to estimate. Accordingly, various modifications were tried in attempts to produce a more satisfactory stock solution, of which the following is recommended.

Production of Standards.

1. Stock Solutions of Acid Hematin.—Defibrinated or oxalated blood is centrifuged, the serum removed, sufficient water added for laking, and hydrochloric acid added to a concentration of 0.1 N. This is allowed to stand for 24 hours at room temperature for full color development. The solution formed corresponds very well to that prepared by the method of Cohen and Smith, or Robscheit, but the absence of the greater part of the serum proteins favors concentration as described later. The solution so prepared is distinctly turbid, but by repeated filtration, preferably through hardened papers, a perfectly clear solution is obtained, having a concentration of from 7 to 12 per cent depending upon that of the unfiltered preparation, and showing only a very slight degree of sedimentation even after standing for a week.

Still more concentrated solutions, however, are desirable both from the standpoint of stability and convenience. Since heating is injurious, concentration is best accomplished by evaporation with an electric fan, the solution being poured into a flat dish. The process requires several hours and should be carried out in as dust-free an atmosphere as possible. When the solution has reached a syrupy consistency it is again filtered, this time by suction, in order to remove any dust particles taken up during evaporation. Evaporation is continued to the thickest solution that can be measured with a pipette, usually corresponding to from 15 to 20 per cent according to Haldane's scale. A known dilution is then standardized

E. H. Terrill 181

against a known acid hematin solution and the concentration of the stock solution calculated. It is then diluted to a convenient point, preferably not below 15 per cent, and for later convenience accurately measured into 2 cc. brown glass ampules. Care must, of course, be taken, owing to the viscosity of the solution, to allow the pipette to drain very slowly.

Preservation.—The more highly concentrated solutions require no preservative, some specimens having stood in the laboratory for several months without decomposition. More dilute solutions may show a growth of mold after long standing, but this can be prevented by the addition of from 10 to 20 per cent of glycerol.

TABLE I.

Percentages of Color Retained by Stock Solutions after Sealing in Ampules.

Since sealing.	Color retained.		
	Solution 1.	Solution 2.	Solution 3.
mos.	per cent	per cent	per cent
1	98	98	97
2	96	97	97
3	96	97	96
4	95	96	96
5	95	95	95
6	95	95	95
7	94	95	95
8	94	94	94
9	94	94	94
10	93	92	93

Dilutions made from these ampules, of a strength usually employed for colorimetric work, are perfectly clear, and show only the slightest tendency to sedimentation. This may be prevented almost indefinitely by incorporating from 10 to 20 per cent of glycerol in the diluting fluid. The glycerol furthermore serves as an effectual preservative.

Stability.—In order to test the stability of these ampules under varying conditions, some were placed in a refrigerator, and others kept in the dark at room temperature. These were broken at intervals of a month, and tested against an acid hematin solution of known strength. The results shown in Table I are taken from those kept at room temperature, and are expressed in percentages of the strength at the time of sealing.

There was no appreciable difference in the case of those kept in the refrigerator. In both instances, fading, while more rapid during the first few months, continued slowly. At no time was any qualitative alteration of color noted.

2. *Acid Hematin Protein Powder.*—Since all solutions of acid hematin are obviously subject to change, and in consideration of the almost invariably greater stability of pigments in dry form, it seemed logical to assume that if the preparation could be reduced to a dry powder, from which standards could be prepared by weight, an important step toward the stability of stock preparations would be made. Attempts to prepare such a powder by continuing the evaporation of the above solutions, resulted in preparations which although giving perfectly clear solutions with almost no tendency to sedimentation, were at times soluble only with the greatest difficulty, and at others, showed sufficient deviation from the true color to prevent exact matching. The disturbing element seems to be the presence of too much inert material. The following method, which resembles very closely that employed by Williamson (4) for preparing purified solutions of hemoglobin was found satisfactory for its removal.

Preparation.—Several hundred cc. of defibrinated or oxalated blood are centrifuged, the serum is removed, and the cells are washed four times with normal saline solution. The washed cells are mixed with an equal volume of distilled water, sufficient ether is added to produce complete laking, the mixture thoroughly shaken and allowed to stand 10 minutes. It is filtered or centrifuged to remove the stroma of the red cells, first diluting slightly with distilled water if too viscid. The filtrate is a clear dark red liquid still containing variable amounts of protein. This is removed by the addition of an equal volume of aluminum cream prepared according to the method of Tracy and Welker (5), followed by filtration by suction or preferably centrifugalization. Alcohol is then added in small portions with constant shaking, until it constitutes about 20 per cent of the volume. An appreciable precipitate here would indicate that all the serum protein had not been removed, although a slight turbidity almost always results from the action of the strong alcohol before being sufficiently diluted. In either event the solution should be again filtered through a hardened paper, preferably without suction.

Air or oxygen is then blown through the solution until the hemoglobin is completely saturated, after which $\frac{1}{2}$ volume of 0.25 N hydrochloric acid is added in small amounts with constant shaking. Instead of the turbid solution obtained by the addition of acid directly to whole blood, a very dark sometimes syrupy, but perfectly clear product results. At least 24

hours should be allowed for full color development. Under no circumstances should it be warmed, since it may change to a gelatinous mass soluble only with great difficulty after drying.

Evaporation should be carried out in the same manner as above described, and when the consistency of a thick syrup is reached, the solution is again filtered as before through a hardened paper. Evaporation to dryness is then completed as rapidly as possible, the mixture being frequently stirred. If facilities are available, evaporation may be most rapidly completed over phosphorus pentoxide under greatly reduced pressure, a procedure which furthermore precludes any possibility of contamination by dust particles. When thoroughly dry, the entire bulk of the preparation should be very finely powdered and thoroughly mixed, in order to facilitate solution as well as to insure homogeneity. The yield from 500 cc. of normal blood is about 40 gm.

Characteristics.—The resulting product is a dark brown, hard, brittle, vitreous mass, rather easily soluble in distilled water, but much less so in 0.1 N hydrochloric acid. In either instance it forms a perfectly clear solution, the color of which exactly matches that of a freshly prepared solution of acid hematin. It is stable in air, and does not absorb more than 0.1 per cent of moisture when exposed. If perfectly dry, heating to 100°C. for several hours does not affect it, but high temperatures (160°C.) render it insoluble. The age of the oldest batch is now over 3 months, and so far no alteration of any of its properties has been noted.

No attempts have been made at analysis, since the product is obviously a mixture of so called "acid hematin" with the protein of the broken hemoglobin molecule. It has been said that on the conversion of hemoglobin into acid hematin, the latter constitutes approximately 4 per cent, while the protein fraction constitutes 96 per cent of the resulting products. All attempts to remove the protein fraction by precipitation have failed owing to the simultaneous precipitation of the pigment. It is difficult to determine the exact state in which the latter exists, but if we accept the usual statements as to its insolubility in water or hydrochloric acid, we must suppose it to be in colloidal form, the maintenance of which is aided by the relatively large amount of protein. This assumption receives further support from its failure to dialyze through a parchment membrane.

Solutions made with 0.1 N hydrochloric acid differ further from the substance commonly termed "acid hematin" in that even

in concentrated solutions, no distinct bands are visible in its spectrum, but only a diffuse absorption approaching the yellow from both sides.

The most striking characteristic of the product is, however, a remarkable uniformity of color of specimens made at different times from different kinds of blood. One might be led to expect this however, since it is made under identical conditions from comparatively pure solutions of hemoglobin. Although the composition of hemoglobin undoubtedly varies in the types investigated, this does not seem to be of a character or degree sufficient to affect the color of the solution for colorimetric work.

TABLE II.

Comparative Color of Different Preparations of Powder Containing 1 Mg. per Cc.

Blood	Source.	Standard setting.	Unknown reading.
		mm.	mm.
1	Human.	20	20.0
2	Sheep.	20	19 9
3	"	20	19.9
4	"	20	20.0
5	"	20	20.2
6	Dog.	20	20.1

In order to demonstrate this uniformity of color, solutions containing 1 mg. of dry powder per cc. were prepared according to the method to be described later, from six different batches of powder, which had been made at different times, from sheep, dog, and human blood, as indicated in Table II. No. 1 was arbitrarily chosen as a standard with which the others were compared. The results are given in Table II.

It would seem that these results obviate the necessity of checking by Van Slyke's oxygen capacity method.

In order to demonstrate further this constancy of color, two solutions, each containing 1 mg. of the dry powder per cc., were made up as described under "Preparation of standard solutions," from each of five different batches of powder. Each solution was read against 0.5 per cent acid hematin solutions prepared as described under "Procedure for hemoglobin determination," from each of

fifteen different bloods whose hemoglobin had been previously determined by Van Slyke's oxygen capacity method. From these data, calculations were made of the amount of dry powder per cc. of solvent required to give a color corresponding to a 1 per cent solution of acid hematin prepared from blood containing 10 gm. of hemoglobin per 100 cc. The average value was 1.48 mg. per cc., the highest being 1.51, and the lowest 1.46.

It is thus seen that 1.48 mg. of the powder are required to give the same color as 1 mg. of hemoglobin after conversion into acid hematin. One might expect the amounts to be more nearly equal in view of the relative purity of the hemoglobin solution from which the powder is prepared. The cause of this difference is not quite apparent and will require further investigation.

Standardization.—The powder is prepared for standardization by dissolving a carefully weighed portion, usually between 30 and 50 mg., in sufficient distilled water to make a solution containing 2 mg. per cc., and then adding an equal volume of accurate 0.2 N hydrochloric acid. Heating is to be avoided. Owing to slight color changes which may follow the addition of the acid, it is allowed to stand for an hour before reading. This is done against a solution of known strength made from blood previously laked with distilled water as later described. From this comparison, the number of mg. of powder per cc. required to give a solution of any desired strength can be readily calculated.

Preparation of Standard Solutions.—Standard solutions to be used regularly in the laboratory should contain from 10 to 20 per cent of glycerol in order to prevent any slight sedimentation or the possible growth of molds. This is best added directly to the water, and a measured quantity of the mixture used. In order to prepare a standard, the powder is weighed to 0.1 mg. and the required amount of solvent calculated. It is completely dissolved in half this volume of the above mentioned glycerol solution, after which an equal amount of accurate 0.2 N hydrochloric acid is added. After standing an hour it is ready for use. If kept at room temperature in tightly stoppered brown glass bottles, it remains unchanged for from 4 to 6 weeks, but after this, slight fading will have occurred, although deviation from the true color has rarely been noted even after much longer periods. The simplicity of such a method is obvious, and since sufficient material

can be obtained from 500 cc. of blood to supply a large laboratory or hospital for months, frequent preparation and standardization are avoided.

3. *Acid Hematin Films.*—Because of the generally greater stability of dry preparations, attempts were made to prepare transparent films of acid hematin which could be employed in the same manner as Newcomer's glass plate. The following method will be found convenient.

A concentrated aqueous solution is prepared from the powdered acid hematin, and sufficient hydrochloric acid is added to bring the acidity to about 0.1 N. This is added to a moderately thick solution of gelatin previously filtered and cooled to about 60°C. After thorough mixing, with care to avoid the formation of air bubbles, it is poured on carefully cleaned 35×55 mm. coverglasses. After draining off the excess, these are carefully leveled and placed in a dust-free place to dry.

A few trials may be necessary to obtain the optimum color, which may be varied to suit the wishes of the individual without sacrifice of accuracy. Films so made are almost perfectly uniform except at the edges. These can be trimmed off with a diamond, and squares of any desired size cut from the remainder. They are mounted in balsam on thin white microscope slides and standardized against a known acid hematin solution. A factor can easily be computed for each film, expressing readings either in percentage or grams of hemoglobin.

Compared with the colored glass plate of Newcomer, they possess the decided advantage of a much darker color, although matching perfectly that of acid hematin solutions and so allowing greater ease and accuracy of readings. Their chief disadvantage lies as might be expected, in a certain lack of permanence, yet kept in the dark they have retained their full color over a period of several months.

Turbidity.

The effect of turbidity of both standard and unknown, invariably present when prepared by the addition of whole blood directly to 0.1 N hydrochloric acid, does not seem to have been sufficiently appreciated.

It has been found impossible to duplicate the reported close agreements between colorimetric and gasometric methods on bloods of widely divergent hemoglobin content, using acid hematin standards prepared in the usual manner. Cohen and Smith (2) state that they also have found it difficult to obtain close agreement on some bloods. The factor of turbidity, which is *not* proportional to the amount of hemoglobin, is offered as at least one possible explanation of this discrepancy.

TABLE III.

Comparison of Hemoglobin Values by Gasometric and Colorimetric Methods Showing Effect of Failure to Lake Blood.

Blood.	Concentration.	Hemoglobin by colorimeter.				Hemoglobin by oxygen absorption.
		Laked.	Not laked	Difference.		
	per cent	*gm. per 100 cc.*	*gm. per 100 cc.*	*mm.*	*per cent*	*gm. per 100 cc.*
1	0.5	10 0	11 0	1 0	10 0	10 1
2	0.5	14.1	15 2	1 1	7 8	14 3
3	0.5	12.4	13 5	1 1	8 9	12 7
4	0 5	15.2	16 4	1 2	7.9	15 0
5	0.5	9 7	10 6	0 9	9 3	9.7
6	0.5	13.4	14 3	0 9	6 7	13 5
7	0.5	11.0	12 0	1 0	9.1	11 0
8	0.5	8.4	9.4	1 0	11.9	8 5
9	1 0	10 6	10 7	1 1	10 4	10 4
10	1.0	15.8	16 8	1 0	6 3	15 5
11	1.0	13.2	14 1	0 9	6 8	13 0
12	1.0	12.9	13 8	0.9	7 0	12 8

Anyone can easily demonstrate that the addition of whole blood directly to 0.1 N hydrochloric acid gives values from 6 to 12 per cent higher than those obtained from the same blood first laked by adding it to distilled water. It is obvious that the higher readings can only be due to turbidity, since all other factors are equal.

In order to demonstrate this, two 0.05 cc. samples were taken in the same pipette from the same specimen of blood. One was delivered directly into 10 cc. of 0.1 N hydrochloric acid, and the other into 5 cc. of distilled water contained in accurate 10 cc. graduates. To the latter after complete laking, which requires

about $\frac{1}{2}$ minute, 0.2 N hydrochloric acid was added to the 10 cc. mark. After standing 24 hours these were read against an acid hematin standard prepared from the powder, corresponding to a 1 per cent solution made from blood containing 10 gm. of hemoglobin per 100 cc. When readings are made with this standard against 20 mm. of the unknown, the depth in mm. is equal to the number of grams of hemoglobin per 100 cc. of the unknown blood. The results in Table III are so expressed.

It is apparent from Table III that the difference in readings on laked and unlaked bloods tends to be constant, rather than to bear a relation to the amount of hemoglobin. Using the unknown as a 0.5 per cent solution prepared by adding directly to 0.1 N hydrochloric acid, the average increase of color is seen to equal 1 mm. of the standard, or 1 gm. of hemoglobin per 100 cc. Employing the unknown as a 1 per cent solution, the difference is the same. But if the standard be of equal turbidity, which seems to be the case when similarly prepared, the error due to this factor approaches zero as the hemoglobin values approach one another.

It has been found that if the blood is first laked by adding it to an amount of distilled water equal to half the required final volume, then making up to volume with 0.2 N hydrochloric acid, that turbidity is so greatly reduced as to become scarcely discernible. By this means the variations between the colorimetric and gasometric methods may be brought within the percentage of error of colorimetric readings.

Effect of Variation of Temperature and Acid Concentration.

The effect of variations of temperature and acid concentration have been discussed by Stäubli (6), Newcomer (7), and Meulengracht (8), who offered certain alterations of technique tending toward increased accuracy. All present curves of color development, but that of Newcomer determined by spectrophotometric methods, presumably using 0.1 N hydrochloric acid at room temperature, is probably the most accurate. Berman (9) recommends boiling the acid hematin solution for 1 minute to develop the maximum color.

The employment of heat, while perhaps applicable to the Sahli hemometer for which it was recommended, is scarcely

It is remarkably constant in composition and for ordinary use, if properly prepared, need not necessarily be checked by Van Slyke's gasometric method.

Although an accurate balance is required for the preparation of standards, these need not be made except at infrequent intervals provided they are properly preserved. Known amounts of powder may be readily sealed in glass containers, and these broken when desired. This would allow one to employ the method clinically where even occasional access to a balance is possible.

Solutions prepared as indicated are of unvarying accuracy and can be duplicated at will. Furthermore, it is possible to prepare them of any desired strength by simple calculation. They may be used in any colorimeter, and are especially applicable to the wedge type owing to the absence of sedimentation. They are more stable than when prepared in the usual manner, and their permanent transparency is an undeniable advantage for all colorimetric work.

The additional step of preliminary laking of the blood sample before adding it to the acid is advisable both in standardization and routine determinations, since the time required is negligible and closer agreement of the colorimetric and gasometric methods on bloods of widely different hemoglobin content is assured.

Procedure for Hemoglobin Determinations.—A pipette containing 50 c.mm., which can readily be filled from a finger prick has been found most suitable. This is filled, the blood thoroughly mixed with 5 cc. of distilled water in an accurate 10 cc. graduate, and the pipette rinsed. After standing $\frac{1}{2}$ minute to insure complete laking, 0.2 N hydrochloric acid is added to the 10 cc. mark, making a 0.5 per cent solution of acid hematin. After standing 10 to 15 minutes it may be read. To read, the unknown is placed on the left side of the Duboscq colorimeter and set at 20 mm., the readings being made with the standard.

Ordinarily a liquid standard is preferred to the gelatin film, since it permits the use of any desired depth of color. A solution corresponding to 10 gm. of hemoglobin per 100 cc. has been found most desirable, for when the unknown is made up as a 0.5 per cent solution as above described, and set at 20 mm., readings being made with the standard, the depth of the latter in mm. is equal to the number of grams of hemoglobin per 100 cc. of the unknown

blood. Corrections may then be made from Newcomer's equation, the time since the addition of the acid being known.

This method has been used in a large number of determinations, and can be recommended as practicable, convenient, and accurate.

SUMMARY.

1. Methods are offered for preparing: (*a*) A concentrated stock solution of acid hematin to be kept in ampules; (*b*) a stable dry acid hematin protein powder of uniform color, from which standards may be prepared by weight; (*c*) transparent acid hematin gelatin films which may be substituted for Newcomer's glass plate.

2. The factor of turbidity invariably present in acid hematin solutions as usually prepared from whole blood is discussed, and suggestions are offered for its elimination.

3. The employment of heat for the more rapid development of maximum color has been found objectionable.

4. A method suitable for routine hemoglobin determinations is proposed.

BIBLIOGRAPHY.

1. Van Slyke, D. D., and Stadie, W. C., *J. Biol. Chem.*, 1921, xlix, 1.
2. Cohen, B., and Smith, A. H., *J. Biol. Chem.*, 1919, xxxix, 489.
3. Robscheit, F. S., *J. Biol. Chem.*, 1920, xli, 209.
4. Williamson, C. S., *Arch. Int. Med.*, 1916. xviii, 505.
5. Tracy, G., and Welker, W. H., *J. Biol. Chem.*, 1915, xxii, 55.
6. Stäubli, C., *Münch. med. Woch.*, 1911, lviii, 2429.
7. Newcomer, H. S., *J. Biol. Chem.*, 1919, xxxvii, 465.
8. Meulengracht, E., *Folia Hæmatol.*, 1921–22, xxvii, 1.
9. Berman, L., *Arch. Int. Med.*, 1919, xxiv, 553.

THE FATE OF CERTAIN SULFUR COMPOUNDS WHEN FED TO THE DOG.*

By CARL L. A. SCHMIDT and GUY W. CLARK.

(*From the Department of Biochemistry and Pharmacology, the University of California, Berkeley.*)

It is a generally accepted fact that the synthesis of the sulfur-containing bile acids proceeds according to the following equations:

$$H_2CS \cdots\cdots SCH_2 \quad CH_2SH \quad CH_2SO_2OH$$
$$HCNH_2 \quad CHNH_2 \rightarrow CHNH_2 \rightarrow CHNH_2 \rightarrow$$
$$COOH \quad COOH \quad COOH \quad COOH$$
$$\text{(Cystine)} \qquad \text{(Cysteine)} \quad \text{(Cysteic acid)}$$

$$CH_2SO_2OH$$
$$CH_2NH_2 \longrightarrow \text{Taurocholic, etc. (bile acids)}$$
$$\text{(Taurine)}$$

The cystine used by the body for the synthesis of bile acids is derived from the common protein catabolism and includes both endogenous and exogenous factors (1). Taurine appears to be available for combination with cholic acid at all times and hence is not the limiting factor which determines the amount of bile acids which the body can synthesize. Our experimental work with the above as well as with certain closely related compounds has been restricted to the determination of the ability of the body to oxidize the sulfur to sulfates and to utilize the nitrogen. We have also been interested in the relative rates of excretion of the nitrogen and of the sulfur of cystine and in the possible conjugation of taurine with urea to form taurocarbamic acid. The substances studied afford an excellent opportunity for the correlation of chemical structure with fate in the body. The demands of the

193

body for sulfur are probably entirely supplied by cystine and not by the ingestion of other substances which we have administered to the dog. However, certain of the compounds, particularly taurine (2), occur as normal constituents of food products and are ingested in small quantities.

The experiments of Salkowski (3), Smith (4), and Heffter (5) with respect to the ability of the dog to oxidize the sulfur which is contained in sulfonic acids to sulfuric acid are at variance, and similarly, the statement that thiosulfuric acid appears in the urine when cystine is fed (6) is not confirmed by the experiments of all who have worked on this subject (7). Experiments which were carried out in this laboratory by Schmidt (8) and his co-workers show clearly that when taurine is ingested by man there is no marked increase in urinary sulfates and that contrary to the reports of Salkowski taurine is not excreted as taurocarbamic acid but as free taurine (9).

EXPERIMENTAL.

The experimental work was carried out on dogs which were kept on constant diets containing a low but not a minimal amount of protein. The dosage of the particular substance given was added to a part of the food which was fed first. Certain of the animals, particularly Dog 1, consumed the food rapidly, the others more slowly. Urine collections were made in a metabolism cage in the usual manner and the dogs were catheterized at the end of the 24 hour period. Estimations of the sulfur fractions were carried out with the aid of the methods of Folin (10) and of Denis (11) and the amino nitrogen estimations were performed with the aid of Van Slyke's method (12). Cysteic acid was prepared from cystine according to the method described by Friedmann (13). Taurine was obtained from the abalone (*Haliotis*) by the method described by Schmidt and Watson (2). For the preparation of isethionic acid, taurine was treated with an excess of nitrous acid as suggested by W. Gibbs (14), and after acidifying and evaporating the solution, the residue was extracted with absolute alcohol from which, on cooling, the product crystallizes.

The results are given in Tables I to VI and a résumé, showing the significant figures of these experiments, is contained in Table VII. In the interpretation of the data, due consideration must be given

TABLE I.
Experiment I.   Dog 1.

Day of experiment	Total sulfur.	Inorganic sulfur.	Ethereal sulfur.	Neutral sulfur.	Total nitrogen.	α-amino nitrogen.	Remarks.
	gm.	gm.	gm.	gm.	gm.	mg.	
1	0.291	0.159	0 030	0 103	4 23	170	Weight 13 9 kilos.
2	0.254	0.129	0 012	0.123	3.70	118	
3	0.206	0.121	0.010	0 075	3.36	90	
4	1.277	0.145	0 015	1.117	4 20	493	5 85 gm. taurine = $\begin{cases} 1.5 \text{ gm. S} \\ 0.66 \text{ gm. N} \end{cases}$
5	0.328	0.152	0 022	0 154	3.46	134	
6	0.827	0.212	0 014	0.602	3.81	166	6.7 gm. cysteic acid as Na salt = $\begin{cases} 1.123 \text{ gm. S} \\ 0.49 \text{ gm. N} \end{cases}$
7	0.298	0.085	0.006	0.207	3 33	144	
8	0.915	0.178	0.013	0 724	3 78	178	7 0 gm. cysteic acid as Na salt = $\begin{cases} 1.171 \text{ gm. S} \\ 0.510 \text{ gm. N} \end{cases}$
9	0.342	0 075	0 010	0.256	3.28	105	
10	1.120	0.185	0 010	0 925	4 10	184	3 7 gm. isethionic acid* as Na salt = 0.795 gm. S. Slight diarrhea, also diuresis.
11	0.746	0.111	0.013	0.622	3.76	157	Soft stools.
12	0.637	0.134	0 014	0.489	3 44	141	
13	0.680	0.120	0 015	0 545	3.15	100	2 8 gm. isethionic acid* as Na salt = 0 613 gm. S.
14	0.359	0.131	0.012	0.215	3.33	94	
15	1.452	0.150	0.012	1.291	3.28	103	8 2 gm. isethionic acid† as Na salt = 1 766 gm. S. Inorganic S = 0 012 gm.
16	0.244	0.121	0.016	0.107	3 55	108	
17	1.371	0.698	0.016	0.656	4 06	301	7.5 gm. cystine = $\begin{cases} 2.00 \text{ gm. S} \\ 0.878 \text{ gm. N} \end{cases}$
18	0.675	0.213	0.020	0.443	3.80	253	Soft stools.
19	0.298	0.185	0.013	0.100	3 36	90	
20	0.275	0.142	0.007	0 126	3.56	122	7 gm. taurocholic acid = $\begin{cases} 0.441 \text{ gm. S} \\ 0.195 \text{ gm. N} \end{cases}$
21	0.244	0.142	0.009	0.094	3.74	106	Soft stools. Weight 15.9 kilos.

* Prepared from taurine by treatment with HNO_2.

† Kahlbaum preparation—50 per cent aqueous solution.

Daily Diet.

Cracker meal.. 200 gm.

Crisco... 40 "

Sucrose.. 60 "

Fullers' earth... 50 "

Condensed milk... 100 cc.

Distilled water sufficient to make a "stiff dough."

TABLE II.

Experiment 2.   Dog 2.

Day of experiment.	Total sulfur.	Inorganic sulfur.	Ethereal sulfur.	Neutral sulfur.	Total nitrogen.	α-amino nitrogen.	Remarks.
	$gm.$	$gm.$	$gm.$	$gm.$	$gm.$	$mg.$	
1	0.103	0.042	0.009	0.052	3.28	49	Weight 12.7 kilos.
2	0.103	0.053	0.009	0.041	3.11	60	
3	0.097	0.047	0.008	0.042	2.86	50	7.5 gm. cystine = $\begin{cases} 2.0\ \text{gm. S} \\ 0.878\ \text{gm. N} \end{cases}$
4	0.919	0.752	0.005	0.163	3.30	109	
5	0.267	0.164	9.005	0.098	2.90	79	
6	0.147	0.077	0.010	0.061	2.55	77	
7	0.712	0.073	0.007	0.632	2.76	63	11 cc. of Kahlbaum's 50 per cent isethionic acid as Na salt = 1.035 gm. S.*
8	0.118	0.039	0.007	0.073	2.87	66	
9	0.079	0.023	0.009	0.048	2.54	50	
10	0.526	0.080	0.006	0.440	2.76	55	5.8 gm. cysteic acid as Na salt = $\begin{cases} 0.989\ \text{gm. S} \\ 0.425\ \text{gm. N} \end{cases}$
11	0.189	0.133	0.010	0.046	2.64	67	
12	0.125	0.015	0.006	0.104	2.67	56	6 gm. taurine = $\begin{cases} 1.54\ \text{gm. S} \\ 0.67\ \text{gm. N} \end{cases}$
13	1.203	0.051	0.005	1.147	3.03	515	
14	0.262	0.021	0.008	0.233	2.87	84	
15	0.146	0.036	0.007	0.102	2.52	76	
16	0.125	0.030	0.012	0.083	3.05	65	
17	0.111	0.028	0.006	0.077	2.50	68	7.5 gm. cystine = $\begin{cases} 2.0\ \text{gm. S} \\ 0.878\ \text{gm. N} \end{cases}$
18	0.888	0.708	0.006	0.174	3.10	155	
19	0.317	0.134	0.017	0.166	2.60	95	
20	0.224	0.104	0.009	0.111	2.80	46	
21	0.152	0.048	0.034	0.070	2.80	68	

* Contains 0.013 gm. S as sulfate.

Diet.

Cracker meal	175 gm.
Crisco	25 "
Sucrose	80 "
Fullers' earth	40 "
Condensed milk	90 cc.
20 per cent beef extract	10 "

to variability for it is difficult in experiments of several weeks duration to eliminate daily fluctuations in the output of urinary constituents and the selection of normal values is difficult.   In the résumé the normal values were obtained either by taking the average of several days of the fore period or by using the figures preceding the experimental day.

TABLE III.

Experiment 3.   Dog 2.*

Day of experiment.	Urinary sulfur.			Urinary nitrogen.		Remarks.
	Total.	Inorganic.	Neutral plus ethereal.	Total.	α-amino nitrogen.	
	gm.	gm.	gm.	gm.	mg.	
1	0.398	0.113	0.185			Weight 14.5 kilos.
2	0.247	0.106	0.141			
3	0.671	0.473	0.198			2 gm. $Na_2S_2O_3 \cdot 5H_2O = 0.516$ gm. S†
4	0.179	0.082	0.097			
5	0.537	0.426†	0.111			1.5 gm. $Na_2SO_3 = 0.381$ gm. S‡§
6	0.149	0.071	0.078			
7	0.404	0.300	0.104			1 gm. $Na_2S_2O_3 \cdot 5H_2O = 0.258$ gm. S‖
8	0.155	0.075	0.080	2.49	52	
9	0.946	0.765	0.181	3.21	75	7.5 gm. cystine = $\begin{cases} 2.0 \text{ gm. S} \\ 0.878 \text{ gm. N} \end{cases}$
10	0.361	0.196	0.165	2.48	59	

* About a month elapsed between Experiments 2 and 3, during which time the dog had been returned to the animal house and received a meat and bread diet.
† When the urine was acidified with H_3PO_4 and distilled, S and SO_2 were obtained in large amounts.
‡ 0.046 gm. S was present as sulfate.
§ Distillation shows only traces of sulfites in the urine.
‖ Urine contains only a small amount of thiosulfate.

Diet.

Cracker meal	150	gm.
Crisco	20	"
Sucrose	65	"
Fullers' earth	40	"
Condensed milk	75	cc.
20 per cent beef extract	10	"

TABLE IV.

Experiment 4.　Dog 3.*

Day of experiment.	Urinary sulfur.			Total nitrogen.	Remarks.
	Total.	Inorganic plus ethereal.	Neutral.		
	gm.	gm.	gm.	gm.	
1	0 110	0 074	0 036	2 50	Weight 11.4 kilos.
2	0 126	0 083	0 043	2 68	
3	0 112	0 072	0 040	2.38	
4	1 084	0.799	0.285	2 77	7 gm. cystine = $\begin{cases} 0.819 \text{ gm. N} \\ 1.87 \text{ gm. S} \end{cases}$
5	0 241	0 186	0 055	2 02	
6	0 136	0 095	0 041	1 96	

* Young growing dog.

Diet.

Cracker meal... 75 gm.
Crisco.. 15 "
Sucrose... 30 "
Fullers' earth.. 25 "
Condensed milk... 50 cc.

TABLE V.

Experiment 5.*　Dog 4 (13.5 kilos).

Day of experiment.	Total sulfur.	Inorganic sulfur.	Ethereal sulfur.	Neutral sulfur	Total nitrogen.	Remarks.
	gm.	gm.	gm.	gm.	gm.	
1	0 135	0 091	0.005	0 039	2 50	
2	0 164	0 104	0 009	0 051	2 51	
3	0 180	0 118	0 007	0 055	2 58	0 210 gm. S as purified taurocholic acid given by mouth.
4	0 168	0 101	0 004	0 063	2 42	
5	0 166	0 097	0 007	0 061	2 40	

* Experiments 5 and 6 were carried out in this laboratory by Mr. Thomas Watson.

Daily Diet.

Lard... 30 gm.
Butter... 10 "
Cracker meal... 200 "
Sugar.. 60 "
Milk... 100 cc.
Kaolin... 50 "

With cysteic acid there is an increase neither in urinary sulfates nor in amino nitrogen[1] which indicates that deamination took place but that the remainder of the molecule was apparently excreted unchanged. Neither sulfurous nor thiosulfuric acid was found in the urine. In contrast to these findings are the results which were obtained on feeding taurine. The amino nitrogen figures indicate that taurine does not combine with urea to form taurocarbamic acid and the sulfur values indicate that there is no appreciable oxidation of the sulfur. These facts agree with the previous findings of Schmidt and Allen on man (9). Cysteic acid, which contains an amino group in a position alpha

TABLE VI.

Experiment 6.* Dog 4 (13.5 kilos).

Day of experiment.	Total sulfur.	Inorganic sulfur.	Ethereal sulfur	Neutral sulfur.	Total nitrogen.	Remarks.
gm.	gm.	gm.	gm.	gm.	gm.	
1	0.175	0.095	0.012	0.068	2.70	
2	0.177	0.098	0.012	0.067	2.66	
3	0.171	0.096	0.009	0.066	2.62	
4	0.945	0.852	0.020	0.073	3.76	S 7 gm. cystine by mouth = $\begin{cases} 2.32 \text{ gm. S} \\ 1.01 \text{ gm. N} \end{cases}$
5	0.456	0.430	0.013	0.013	2.56	
6	0.187	0.098	0.011	0.078	2.58	
7	0.175	0.096	0.011	0.068	2.68	

* The daily diet was the same as in Experiment 5.

to the carboxyl group, is deaminized on passage through the body despite the fact that it also contains a sulfonic group, while taurine, which reacts *in vitro* in all respects like an α-aminocarboxylic acid, is not so deaminized. The replacement of the amino group of taurine by a hydroxyl group to give isethionic acid does not lead to an increase in urinary sulfates. A marked increase in the output of both sulfur and nitrogen coincident with the administration of the first dose of isethionic acid to Dog 1 was found. Apparently a factor other than the substance administered was responsible for the increase in endogenous metab-

[1] Cysteic acid yields its nitrogen quantitatively when treated with HNO_2.

TABLE VII.

Résumé Showing the Urinary Recovery of the Substances Ingested.*

Experiment 1.

Substance ingested.	Inorganic sulfur. Experimental value. gm.	Normal value. gm.	Recovery. per cent	Neutral sulfur. Experimental value. gm.	Normal value. gm.	Recovery. per cent	Total sulfur. Experimental value. gm.	Normal value. gm.	Recovery. per cent	Total nitrogen. Experimental value. gm.	Normal value. gm.	Recovery. per cent	α-amino nitrogen. Experimental value. mg.	Normal value. mg.	Recovery. per cent
Taurine..........	No increase.			1.117	0.117	67	1.277	0.263	68	4.20	3.50	106	493	114	58
Cysteic acid 1. Output 1st day......	0.212	0.141	6	0.602	0.117	43	0.827	0.263	50	3.81	3.50	63	No increase.		
" 2nd "				0.207	0.117	8	0.298	0.263	3						
						51			53						
Cysteic acid 2. Output 1st day......	0.178	0.141	3	0.724	0.117	52	0.915	0.263	56	3.78	3.50	74	No increase.		
" 2nd "				0.256	0.117	12	0.342	0.263	6						
						64			62						
Isethionic acid 1. Output 1st day......				0.925	0.489	55	1.120	0.637	61†						
" 2nd "				0.622	0.489	17	0.746	0.637	14						
						72			75						

Isethionic acid 2.............				0.545	0.215	54		0.680	0.359	52		
" 3.............				1.291	0.107	61		1.452	0.244	68		
Cystine.												
Output 1st day...............	0.698	0.156	27	0.656	0.107	28	1.371	0.272	55	4.06	3.55	58	301	110	22
" 2nd "	0.213	0.156	3	0.443	0.107	17	0.675	0.272	20	3.80	3.55	28	253	110	16
			30			45			75			86			38
Taurocholic acid.§												
Output 1st day.‡...............	0.698	0.156	27				0.822	0.272	28	3.87	3.55	36
" 2nd " .‡...............	0.213	0.156	3				0.339	0.272	3	3.66	3.55	12
			30						31			48

Experiment 2.

Cystine.												
Output 1st day...............	0.752	0.047	35	0.163	0.045	6	0.919	0.101	41	3.30	2.90	46	109	53	6
" 2nd "	0.164	0.047	6	0.098	0.045	3	0.267	0.101	8	No increase.		
" 3rd "	0.077	0.017	2	0.061	0.045	1	0.147	0.101	2	"		
			43			10			51			

* Blank spaces indicate that the output values are within the limits of the normal daily variation.

† The abnormally high values for the output of nitrogen and sulfur were apparently due to factors other than the ingestion of isethionic acid. The sulfur values of the 2nd day following the ingestion of isethionic acid are taken as normal values for the purpose of calculating sulfur output.

‡ The calculations are on the basis of catabolized cystine.

§ The figures in Table I indicate that taurocholic acid is not eliminated in the urine.

TABLE VII.—*Concluded.*

Experiment 2—*Concluded.*

Substance ingested	Inorganic sulfur. Experimental value. *gm.*	Inorganic sulfur. Normal value. *gm.*	Inorganic sulfur. Recovery. *per cent*	Neutral sulfur. Experimental value. *gm.*	Neutral sulfur. Normal value. *gm.*	Neutral sulfur. Recovery. *per cent*	Total sulfur. Experimental value. *gm.*	Total sulfur. Normal value. *gm.*	Total sulfur. Recovery. *per cent*	Total nitrogen. Experimental value. *gm.*	Total nitrogen. Normal value. *gm.*	Total nitrogen. Recovery. *per cent*	α-amino nitrogen. Experimental value. *mg.*	α-amino nitrogen. Normal value. *mg.*	α-amino nitrogen. Recovery. *per cent*
Isethionic acid.															
Output 1st day........				0.632	0.045	57	0.712	0.101	59						
" 2nd "				0.073	0.045	3 | 60	0.118	0.101	1 | 60						
Cysteic acid.															
Output 1st day........	0.133	0.050	10	0.140	0.046	40	0.526	0.125	41	2.76	2.50	40	No increase.		
" 2nd "							0.189	0.125	6 | 47						
Taurine.															
Output 1st day........				1.147	0.102	68	1.203	0.146	69	3.03	2.70	49	515	76	66
" 2nd "				0.233	0.102	9 | 77	0.263	0.146	8 | 77	2.87	2.70	25 | 74			
Cystine.															
Output 1st day........	0.708	0.048	33	0.174	0.070	5	0.888	0.152	31	3.10	2.60	57	155	68	10
" 2nd "	0.134	0.048	4	0.166	0.070	5	0.317	0.152	8				95	68	3 | 13
" 3rd "	0.104	0.048	3 | 40	0.111	0.070	2 | 12	0.224	0.152	4 | 46						

Experiment 3.‖

Sodium thiosulfate	0.473	0.076	77	0.198	0.085	22	0.671	0.168	98		
" sulfite	0.426	0.076	92	0.117	0.085	8	0.537	0.168	97		
" thiosulfate	0.300	0.076	87	0.104	0.085	7	0.404	0.168	92		
Cystine. Output 1st day	0.765	0.076	35	0.181	0.085	5	0.946	0.168	39	3.21 2.48 83
" 2nd "	0.196	0.076	6	0.165	0.085	4	0.361	0.168	10	
			41			9			49	

Experiment 4.¶

Cystine. Output 1st day	0.799	0.076	39	0.285	0.041	13	4.084	0.116	52	2.77 2.20 70
" 2nd "	0.186	0.076	6				0.241	0.116	7	
			45						59	

Experiment 6.

Cystine. Output 1st day	0.852	0.096	33		0.915	0.175	33	3.76 2.62 413
" 2nd "	0.430	0.096	14		0.156	0.175	12	
			47				45	

‖ In this experiment the figures given in the neutral sulfur column are neutral plus ethereal sulfur.

¶ In this experiment the figures given in the inorganic sulfur column are for total sulfates.

olism since subsequent doses of the same substance to this animal and to another dog did not show the effect.

No appreciable amount of thiosulfuric acid was found in the urine following the ingestion of either cysteic acid, taurine, or isethionic acid. This raises the question why other experimenters found thiosulfates on feeding compounds containing sulfonic acid radicals. It is possible that bacterial action was a factor concerned in their experiments. The presence of small quantities of sulfurous or thiosulfuric acid in urine is easily determined by acidifying the urine with H_3PO_4 and distilling in an atmosphere of CO_2. Our experiments with sodium sulfite and sodium thiosulfate indicate that if such substances as isethionic acid and taurine are reduced to sulfurous acid and this compound is absorbed, oxidation is both complete and rapid.[2] If large quantities of thiosulfuric acid are formed from the administered substance by bacterial action and absorption subsequently takes place the urine of the animal will contain thiosulfuric acid, the amount depending on the dosage of thiosulfate which is absorbed. Our experiments with sulfites and thiosulfates are in agreement with the results of Salkowski (15), Lasch (16), and Rost and Franz (17), and show that moderate doses are readily oxidized to sulfates.

Administration of a 7.5 gm. dose of cystine to Dog 1 not only led to a considerable oxidation (30 per cent) of the amino-acid as seen from the increase in urinary sulfates, but curiously, about one-half of the amount administered was excreted in the urine unchanged, the elimination of cystine extending over a period of 2 days. The figures for both the neutral sulfur and the amino nitrogen support this statement and the presence of cystine in the urine was established by subsequent isolation with the aid of Gaskell's method (18). The elimination of unaltered cystine in the urine of Dog 1 was probably due to unusually rapid absorption but it is by no means clear why the elimination of free amino-acid extended over the 2nd day. Similar doses of cystine administered to Dogs 2 (Experiments 2 and 3), 3 (Experiment 4), and 4 (Experiment 6) were followed by minimal increases in neutral sulfur. The major portions of the absorbed cystine were oxidized as evidenced by a very marked increase in the elimination of urinary

[2] Unpublished investigations with sulfurous acid carried out by one of us (C. L. A. S.) on man gave similar results.

sulfates.   Ingestion of 7.5 gm. of cystine did not lead to the appearance of either sulfurous or thiosulfuric acid in the urine of the experimental animals.

From the results of Experiments 1, 2 (second dose), 3, 4, and 6 (see also résumé table) it will be noted that following the ingestion of cystine the magnitude of the figures for nitrogen elimination is larger than the magnitude of the corresponding sulfur figures. From this list we may possibly eliminate the comparison of the nitrogen and sulfur figures of Experiments 1, 2 (second dose), and 4 as being not far outside of the limits of the normal variability but the difference in the percentages of cystine nitrogen and sulfur recovered in the urine as found in Experiments 3 and 4 cannot be so eliminated.   If the difference is due to a specific demand on the part of the animal for anabolic sulfur a retention of both nitrogen and sulfur would be expected since sulfur is built into the protein molecule in the form of cystine.   It is possible that we are dealing with a phenomenon analogous to that studied by Folin and Denis (19).   They found that as a result of bacterial action on the nitrogenous material which has passed into the large intestines the ammonia content of the portal blood is increased.   If cystine is decomposed by bacterial action to NH_3 and H_2S the former can by absorption enter the blood stream while the latter may be eliminated through the intestines.

Our results also show that the elimination of nitrogen after ingestion of cystine precedes that of sulfur.   This fact is shown in Experiments 2, 3, 4, and 6.   Certain of the figures for the output of sulfur on the 2nd and 3rd day after ingestion of cystine probably lie within the variability of the normal value; all, however, point in the same direction.   The lag in the excretion of sulfur is apparently not due to a lag in the elimination of sulfates since the experiment with Na_2SO_3 indicates very prompt elimination.   It appears to us more reasonable to assume that deamination of the cystine precedes the oxidation of sulfur, with the result that the elimination of sulfur lags behind the nitrogen.   This explanation is in accord with the idea of Lewis (20) that the breaking up of the cystine molecule is dependent upon and takes place subsequent to deamination.

A negative Pettenkofer test for bile acids and normal values for urinary sulfur and nitrogen were obtained following the in-

gestion of taurocholic acid and indicate that the urine is not a channel for the elimination of bile acids. Even in icterus the amount of bile acids eliminated in the urine per 24 hours does not exceed several hundred milligrams.[3]

SUMMARY.

Experiments were carried out on dogs to determine the fate of certain sulfur compounds with especial reference to those concerned in bile metabolsim.

It was found that taurine is excreted in the urine unchanged and not as taurocarbamic acid as stated by Salkowski. Cysteic acid is deaminized, but the remainder of the molecule is excreted in the urine unchanged. Administration of isethionic acid is not followed by an increase in urinary sulfates as stated by several investigators. The urine is not a channel for the elimination of bile acids when the latter are fed. When given in large doses the major portion of the absorbed cystine is oxidized to sulfates which are eliminated in the urine. With the exception of large doses of sodium thiosulfate the ingestion of the various sulfur compounds under consideration did not lead to the appearance of appreciable amounts of sulfurous or thiosulfuric acid in the urine.

BIBLIOGRAPHY.

1. Foster, M. G., Hooper, C. W., and Whipple, G. H., *J. Biol. Chem.*, 1919, xxxviii, 379.
2. Schmidt, C. L. A., and Watson, T., *J. Biol. Chem.*, 1918, xxxiii, 499.
3. Salkowski, E., *Virchows Arch. path. Anat.*, 1873, lviii, 460, 580; 1876, lxvi, 315; *Arch. ges. Physiol.*, 1871, iv, 91; 1886, xxxix, 209; *Ber. chem. Ges.*, 1872, v, 637; 1873, vi, 744, 1312.
4. Smith, W. J., *Z. physiol. Chem.*, 1893, xvii, 1, 459; *Arch. ges. Physiol.*, 1893, liii, 481; 1893–94, lv, 542; 1894, lvii, 418.
5. Heffter, A., *Arch. ges. Physiol.*, 1886, xxxviii, 476.
6. Blum, L., *Beitr. chem. Physiol. u. Path.*, 1903–04, v, 1. Wohlgemuth, J., *Z. physiol. Chem.*, 1903–04, xl, 81. Rothera, C. H., *J. Physiol.*, 1904–05, xxxii, 175.
7. Abderhalden, E., and Samuely, F., *Z. physiol. Chem.*, 1905, xlvi, 187.
8. Schmidt, C. L. A., von Adelung, E., and Watson, T., *J. Biol. Chem.*, 1918, xxxiii, 501.
9. Schmidt, C. L. A., and Allen, E. G., *J. Biol. Chem.*, 1920, xlii, 55.

[3] Unpublished investigations of Schmidt and Merrill.

10. Folin, O., *J. Biol. Chem.*, 1905–06, i, 131.
11. Denis, W., *J. Biol. Chem.*, 1910, viii, 401.
12. Van Slyke, D. D., *J. Biol. Chem.*, 1913–14, xvi, 125.
13. Friedmann, E., *Beitr. chem. Physiol. u. Path.*, 1902–03, iii, 1.
14. Gibbs, W., *Am. J. Sc.*, 1858, series 2, xxv, 18.
15. Salkowski, E., *Z. physiol. Chem.*, 1914, xcii, 89.
16. Lasch, W., *Biochem. Z.*, 1919, xcvii, 1.
17. Rost, E., and Franz, F., *Zentr. Biochem. u. Biophys.*, 1912–13, xiv, 518.
18. Gaskell, J. F., *J. Physiol.*, 1907–08, xxxvi, 142.
19. Folin, O., and Denis, W., *J. Biol. Chem.*, 1912, xi, 161.
20. Lewis, H. B., and Root, L. E., *J. Biol. Chem.*, 1922, l, 303.

THE ACETONURIA OF DIABETES.*

By ROGER S. HUBBARD and SAMUEL T. NICHOLSON, Jr.

(From the Laboratories of The Clifton Springs Sanitarium, Clifton Springs, New York.)

Both Shaffer (1921) and Woodyatt (1921), in recent articles, have discussed the acetonuria found in diabetes, in starvation, and in abnormal nutrition due to diets low in carbohydrate as if they could be attributed to the same type of metabolic disturbance. This seems to be the conception of a majority of the writers on the subject at the present time, although some do not accept this theory.[1] The outstanding difference between the two conditions is that normal subjects develop acetonuria when deprived of food, while diabetic patients often show less acetonuria during a fast than they did before. Labbé, Labbé, and Nepveux (1921) have explained this phenomenon as follows: Diabetics often receive more food, particularly more fat, than they can burn, and this excess of fat leads to the production of acetone bodies; when this excess of fat is no longer fed, the excretion of the acetone bodies decreases; normal subjects, on the other hand, receive more carbohydrate than they need to burn the fat taken, and the withdrawal of this carbohydrate causes a production of acetone bodies from fat—in the case of complete inanition from body fat—and so apparently we get an entirely different response to starvation from that given by diabetics. This explanation is similar to that suggested by the papers of Shaffer and Woodyatt cited above. Ladd and Palmer (1920-21) have shown that diets which cause moderate increases of acetone excretion by diabetic patients are essentially similar to those reported by Zeller (1914) and Lang (1915) as

---

* The subject matter presented here formed part of a paper read before the American Society of Biological Chemists in December, 1921 (Hubbard, Nicholson, and Wright, 1922).

[1] See Joslin (1917), p. 162.

causing similar increases in normal subjects. In the paper
presented here further evidence is given to support the work of
Ladd and Palmer, and a method for studying differences in the
response of the subject to ingested fat and to fat drawn from the
reserves of the body is suggested.

In a former paper from this institution (Hubbard and Wright,
1922) on the study of the acetonuria developed by normal sub-
jects fed a diet high in fat and low in carbohydrate, the following
formula was proposed for expressing the ketogenic balance of any
diet as a molecular ratio:

$$100 \times \frac{1.5 \text{ (weight carbohydrate } + 25 \text{ per cent weight protein)}}{95 \text{ per cent weight fat}}$$

In this formula, which is based on the assumption that 1 molecule
of glucose will burn 1 molecule of ketogenic material,[2] the factor
1.5 represents the figure by which glucose must be multiplied to
give the molecular equivalent of fatty acid, 25 per cent weight
protein represents the probable amount of glucose which can be
derived from protein above that needed to burn the acetone bodies
which are derived from the same protein, and 95 per cent weight
fat represents the amount of fatty acids which fat yields on hydrol-
ysis. From a study of the acetone found in the urine as compared
with a series of diets expressed in terms of this ratio it was con-
cluded that the value of the ratio was about 80 per cent when the
acetone excretion became normal. Traces of acetone were found
in some cases when the ratio had a higher value than this, but
the figure given seemed to represent the value of the ratio of "the
border-line diet" as accurately as it was possible to determine it.
This "border-line diet" has the same composition as that described
by Woodyatt (1921):

$$2 \times \text{carbohydrate} + \tfrac{1}{2} \text{ protein} = \text{fat}$$

---

[2] Shaffer, in a paper recently read before the American Society of Biolog-
ical Chemists, has stated that the ratio of 1 molecule of glucose to 2 of
fatty acid represents the conditions for complete oxidation of these com-
pounds in the body, rather than a ratio of 1 molecule to 1 molecule on
which Woodyatt's formula and the one in this paper are based. He stated,
however, that a ratio of 1 molecule to 1 molecule gives a margin of safety
which makes it valuable in controlling the actual diets of diabetic patients
(Shaffer, 1922).

It was necessary to introduce certain changes into the expression of the ratio before it could be applied to the study of cases of diabetes. Most of the patients, for at least a part of the time during which they were under observation, received diets which did not maintain metabolic equilibrium. To allow for this fact the following plan was adopted: it was assumed that all the calories not furnished by retained (ingested and not excreted) carbohydrate, or by metabolized protein, were furnished by fat, either by fat taken in the diet or drawn from the reserve supplies of the body; this assumption may not be strictly correct, but it must be nearly so in the case of most diabetic patients, because their supply of glycogen available for combustion has been depleted by the disease. It was impossible to determine exactly how much fat a patient must draw from his reserves in a day, and the following calculation was used to estimate the amount roughly: the basal metabolism was determined, or, in most cases, calculated from the metabolism of normal subjects of the same height and weight, and an excess of 20 per cent over this was figured as the probable requirement of a diabetic patient under treatment, who was taking the mildest possible form of exercise. It has been found that such diets would maintain the body weight of normal subjects under similar conditions (Hubbard and Wright, 1922), and, while some diabetics maintain their weight on a smaller intake than this, the figure, 120 per cent of the basal requirement, seemed as good a first approximation as could be made for the purpose. The calories from carbohydrate utilized by the organism were calculated by multiplying the difference between the carbohydrate intake and the glucose excreted in the urine by 4; the calories derived from protein can be calculated by multiplying the total nitrogen in the urine by 6.25 to convert it into terms of protein, and then multiplying the product by 4 to convert it into calories, or they can be calculated directly from the protein fed if the subject is in nitrogen equilibrium. The sum of the calories derived from carbohydrate and from protein was subtracted from the estimated caloric requirement to give the calories derived from fat, and this difference was divided by 9 to convert it into terms of grams of fat burned by the patient. The mathematical expression for the weight of total fat burned based on these assumptions and calculations is:

$$120 \text{ per cent calories} - 4 \text{ (gm. carbohydrate fed} - \text{gm. urine}$$
$$\frac{\text{sugar)} - 25 \times \text{gm. urine N}}{9} = \text{gm. fat}$$

In the expression

$$100 \times \frac{1.5 \text{ (weight carbohydrate} + 25 \text{ per cent weight protein)}}{95 \text{ per cent weight fat}}$$

the numerator, which represents the antiketogenic material expressed as glucose, must be changed for studying diabetic patients. When glucose is present in the urine, the amount found should be subtracted from the carbohydrate ingested (Shaffer, 1921); when the patient is not approximately in nitrogen equilibrium, the urinary nitrogen should be multiplied by 1.6 $\left(1.6 = \frac{6.25}{4}\right)$ to give the amount of glucose derived from the protein burned greater than that which is necessary for complete combustion of the ketogenic material from the same protein. The mathematical formula for expressing the ketogenic balance of a diet fed to a diabetic similar to the one used in studying the diets of normal subjects (Hubbard and Wright, 1922) is:

$$100 \times \frac{1.5 \text{ (weight carbohydrate} - \text{weight urine sugar} + 1.6 \text{ weight urine N)}}{95 \text{ per cent weight fat}}$$

The amount of fat should be estimated in some such way as that given above, or, if possible, the proportions of the different food-stuffs should be calculated from complete respiratory exchange data.

Seven cases have been selected out of twenty-five studied to illustrate the method. All the patients were receiving a treatment similar to that outlined by Allen (1913) and Joslin (1917) and we believe that the cases selected for presentation took only the food furnished them from the diet kitchen. After a short period of starvation, or, more often, of marked undernutrition, the food furnished was increased slowly. Protein was the food first increased, and the patients were receiving enough of this foodstuff to maintain them in nitrogen equilibrium during the larger part of the time during which they were studied and all were receiving enough of this foodstuff for this purpose when the acetone excretion became normal. This made it possible to base the calcula-

tion on the protein intake without introducing errors in excess
of the experimental ones, and made the results more directly com-
parable with those previously published (Hubbard and Wright,
1922). None of the subjects showed temperatures above normal
during the study. Included in each case report is a table in which
the diet, weight, alveolar carbon dioxide tension, blood sugar, and
the excretion of sugar and of the acetone bodies in the urine is
recorded. The alveolar carbon dioxide tension was determined
by the method of Fridericia (Fridericia, 1914; Poulton, 1915);.
blood sugar by the Benedict (1918) modification of the method of
Lewis and Benedict (1915); and the acetone bodies by a method
recently published (Hubbard, 1921).

A chart has been plotted for each case presented which shows
the relationship of the calories fed to the calculated basal calorie
requirement of a normal subject of the same height and weight;[3]
changes in body weight; the values of the ratios described above
based on the fat taken in the diet and on the probable amount of
fat actually burned by the patient; and the total acetone bodies
found in the urine expressed as acetone. In these charts a cross
has been used to designate a positive sodium nitroprusside test
at certain times when quantitative analyses were not made.

Case 1 had a mild type of diabetes which had lasted only a short time.
A study of the chart shows that 120 per cent of the normal basal require-
ment represented approximately the caloric needs during the period. The
value of the ratio glucose to fat burned appeared to be about 90 per cent
when the first slight increases of acetone excretion occurred. There was
a slight increase in the amount of acetone excreted on Aug. 19 when the fat
intake was increased, but no change was made in the amounts of protein
and carbohydrate in the diet. This observation seems to show that fat
fed as distinct from body fat, may have an effect upon the excretion of
acetone. This finding is in harmony with the findings and theories stated
by Allen, Stillman, and Fitz.[4] Slight temporary increases in the excretion
of acetone were found at other times.

Case 2 had a mild type of the disease which had lasted only for a short
time. Chart 2 shows that 120 per cent of her calculated basal metabolism
represented approximately the caloric needs during the period of study.
The amount of acetone found in the urine was very small when the ratio
glucose to fat burned had a value of 100 per cent, and finally decreased to

[3] The tables given in Lusk's book (Lusk, 1917), pp. 126 to 129, were used
in making these calculations.

[4] Allen, Stillman, and Fitz (1919), Chapter VI, p. 500.

TABLE I

Case 1.

Date	Carbohydrate gm	Protein gm	Fat gm	Calories	Weight kg	Alveolar CO₂ mm	Blood sugar per cent	Volume cc	Sugar per cent	Sugar gm	Acetone gm	β-hydroxybutyric acid gm
1921												
July 19	?	?	?	?	56 7			1,850	5 2	96 2	0 109	
" 20	59	30	3	383			0 390	1,640	4 2	68 8	0 145	
" 21	64	33	0	388				1,200	3 1	39 0	0 274	0.469
" 22	64	33	0	388				1,120	3 5	39 9	0.206	0 374
" 23	64	33	0	388				870	2 3	19 7		
" 24	64	33	0	388		33		850	2 0	17 0		
" 25	64	33	0	388	56 2			640	1 2	8 0		
" 26	64	33	0	388				520	0 3	1 4	0.057	0 051
" 27	64	33	0	388				520	0 5	2 6	0 040	0 053
" 28	64	33	0	388	55 8			760	0 4	2 8	0 072	0 090
" 29	0	0	0	0				600	0	0		
" 30	3	2	0	20		40		1,420	0	0		
" 31	8	10	6	126	55 6			1,370	0	0		
Aug. 1	8	12	6	134		34		1,510	0	0	0.719	2 12
" 2	10	15	10	190				1,300	0	0	0 586	2 02
" 3	12	20	15	263	54 3	31		1,280	0	0	0.925	2 37
" 4	15	26	25	389				1,040	0	0	0 751	1 59
" 5	20	29	29	457				1,100	0	0	0 534	1 08
" 6	20	29	29	457				1,260	0	0		
" 7	20	29	30	166	53 5	31	0 120	830	0	0	0.125	0 713
" 8	25	40	40	620		31		940	0	0	0 353	0 658
" 9	25	48	50	712				1,120	0	0	0 328	0.563
" 10	30	50	50	770	54 3			1,500	0	0	0.266	0 390
" 11	35	58	65	957				940	0	0	0 368	0.433
" 12	40	71	70	1,071				1,070	0	0		
" 13	40	71	70	1,074				1,150	0	0		
" 14	40	70	70	1,070	54 6		0 150	1,240	0	0		
" 15	40	70	70	1,070				960	0	0		
" 16	16	70	71	1,103				1,480	0	0	0.123	0 087
" 17	40	69	75	1,111	53 7			970	0	0	0 073	0 067
" 18	40	69	85	1,201				1,560	0	0	0 125	0 121
" 19	40	70	85	1,205		32		1,260	0	0	0 189	0 370
" 20	40	76	85	1,229				1,280	0	0	0 248	0 113
" 21	40	75	90	1,270	53 8			1,310	0	0		
" 22	40	75	90	1,270				1,380	0	0	0 072	0 065

TABLE I — *Concluded.*

Date.	Diet.				Weight.	Alveolar CO₂.	Urine.					
	Carbohydrate.	Protein.	Fat.	Calories.			Blood sugar.	Volume.	Sugar.		Acetone.	β-hydroxybutyric acid.
1921	gm.	gm.	gm.		kg.	mm.	per cent	cc.	per cent	gm	gm.	gm.
Aug. 23	40	77	90	1,278				1,260	0	0	0 116	0 099
" 24	40	75	100	1,360	53 7		0 140	1,200	0	0	0 185	0 230
" 25	45	75	100	1,380				1,010	0	0	Positive.	
" 26	47	75	95	1,343				1,360	0	0	"	
" 27	50	75	100	1,400				1,160	0	0	"	
" 28	55	77	100	1,419	53 5			1,090	0	0	"	
" 29	55	75	100	1,420				1,200	0	0	Negative.	
" 30	55	76	100	1,424			0 150	1,010	0	0	"	
" 31	55	72	99	1,399	53 5			800	0	0	"	
Sept. 1	55	75	100	1,420				1,300	0	0	"	
" 2	55	75	100	1,420				1,050	0	0	"	
" 3	55	75	100	1,420				1,000	0	0	"	
" 4	55	75	100	1,420	53 0			800	0	0	"	
" 5	55	75	100	1,420				1,080	0	0	"	
" 6	55	75	100	1,420				1,060	0	0	"	
" 7	60	76	105	1,485	53 1			1,260	0	0	"	
" 8	60	76	105	1,489				1,080	0	0	"	
" 9	60	76	104	1,480				1,500	0	0	Positive.	
" 10	60	75	105	1,485			0 140	1,250	0	0	Negative.	
" 11	60	60	102	1,398	53 3			1,110	0	9	Positive.	
" 12	60	75	104	1,478				1,060	0	0	Negative.	
" 13	60	76	105	1,489				1,200	0	0	"	
" 14	60	52	85	1,213	53 3			1,460	0	0	"	
" 15	60	76	104	1,480				1,220	0	0	"	
" 16	54	64	81	1,201				1,340	0	0	Positive.	
" 17	60	76	105	1,489				1,180	0	0	"	
" 18	60	75	105	1,485	52 8			1,030	0	0	Negative.	
" 19	60	75	105	1,485				1,360	0	0	"	
" 20	60	74	104	1,472				1,180	0	0	Positive.	
" 21	60	75	105	1,485	52 8							

Results of the test with sodium nitroprusside and ammonia are given for specimens on which quantitative determinations of the acetone bodies were not made.

Under acetone is listed acetone plus diacetic acid.

Results of all acetone bodies are expressed in terms of acetone.

CHART 1. Case 1.

CHART 2. Case 2.

TABLE II.

Case 2.

Date.	Diet. Carbohydrate	Protein	Fat	Calories.	Weight.	Alveolar CO$_2$.	Blood sugar.	Urine. Volume.	Sugar. per cent	gm.	Acetone.	β-hydroxybutyric acid.
1921	gm.	gm.	gm.		kg.	mm.	per cent	cc.	per cent	gm.	gm.	gm.
July 1	?	?	?	?					2 5		Positive.	
" 2	55	81	63	1,111	54 1		0 225	1,560	1 6	24 9	Negative.	
" 3	64	33	18	550				1,320	1 0	13 0	Positive.	
" 4	64	33	0	388				1,100	0 3	3 0	Negative.	
" 5	64	33	0	388		36		840	0 8	6 9	"	
" 6	64	33	0	388				1,030	0 7	7 2	"	
" 7	0	0	0	0				710	Trace.		Positive.	
" 8	5	3	0	32				520	0	0	"	
" 9	5	9	6	110				720	0	0	0 438	1 95
" 10	8	10	6	126	52 1	33		1,050	0	0	0 776	3 26
" 11	10	20	6	174		32		830	0	0	0 812	3 78
" 12	15	26	11	263				1,010	0	0	1 03	4 36
" 13	33	36	18	438	51 1	29		780	Trace.		1 56	3 30
" 14	23	40	20	432		33		1,480	0	0		
" 15	36	27	0	252		36		1,325	0	0		0 587
" 16	36	27	0	252	50 9	36		1,700	0	0	0 312	0 467
" 17	36	36	10	378		36		1,720	0	0		
" 18	36	36	10	378				1,410	0	0	0 368	0 428
" 19	40	41	20	504		33		1,225	0	0	0 184	0 216
" 20	40	41	20	504	50 7			1,680	0	0	0 616	0 264
" 21	39	40	30	594		33		1,380	0	0	0 230	0 286
" 22	40	45	40	700		34	0 150	1,270	0	0	0 180	0 174
" 23	45	50	50	820				1,560	0	0	0 161	0 168
" 24	50	60	54	926	50 5	34		1,610	0	0		
" 25	50	65	59	991				1,220	0	0	0 026	0 060
" 26	50	65	60	1,000				1,320	0	0	0 168	0 100
" 27	55	70	70	1,130	50 3			1,040	0	0	0 077	0 093
" 28	55	70	80	1,220			0 170	950	0 3	2.9	0 142	0 111
" 29	56	70	80	1,224				1,425	Trace.		0.133	0 040
" 30	27	37	40	616				1,300	0	0	0 073	0 051
" 31	50	65	60	1,000				1,660	0	0		
Aug. 1	50	65	60	1,000		38		1,160	0	0	0 318	0 214
" 2	50	65	59	991				1,200	0	0	0 177	0 486
" 3	50	65	75	1,135	49 5			1,500	0	0	0 122	0 077
" 4	50	65	76	1,144				1,300	0	0	0 128	0 100
" 5	50	65	74	1,120				1,100	0	0	0 105	0 050
" 6	50	65	75	1,135				1,500	0	0		
" 7	50	65	75	1,135	49 8	39 0	150	1,480	0	0		

TABLE II *Concluded.*

	Diet.							Urine.				
Date	Carbohydrate.	Protein.	Fat.	Calories.	Weight.	Alveolar CO₂.	Blood sugar.	Volume.	Sugar.		Acetone.	β-hydroxybutyric acid.
	gm.	gm.	gm.		kg.	mm.	per cent	cc.	per cent	gm.	gm.	gm.
Aug. 8	50	69	85	1,241				1,600	0	0	0.346	0.566
" 9	50	69	84	1,241				1,200	0	0		
" 10	51	71	89	1,289	49.9			1,120	0	0	0.205	0.088
" 11	50	71	85	1,249			0.170	1,440	0	0	0.093	0.061
" 12	50	70	85	1,245				1,200	0	0	0.080	0.071
" 13	50	70	85	1,245				1,300	0	0		
" 14	50	70	85	1,245	49.5			1,450	0	0		
" 15	50	70	85	1,245				1,300	0	0		
" 16	52	69	84	1,240				1,400	0.2	2.8		
" 17	50	70	85	1,245	49.5			1,280	Trace.		0.042	0.037
" 18	50	70	85	1,245				1,320	0	0	0.077	0.063
" 19	50	70	85	1,245				1,290	0	0	0.076	0.057
" 20	50	70	85	1,245				1,170	0	0	0.066	0.062
" 21	50	69	90	1,286	49.6			1,250	0	0		
" 22	50	70	90	1,290				1,320	0	0	0.036	0.026
" 23	50	69	90	1,286				1,620	0	0	0.073	0.054
" 24	51	70	90	1,290	49.3			1,280	0	0	0.101	0.100
" 25	50	70	90	1,286				1,360	0	0	Positive.	
" 26	50	70	90	1,286				1,200	0	0	Negative.	
" 27	55	75	90	1,330				1,280	0	0	"	
" 28	59	75	90	1,350	48.4			1,320	0	0	Positive.	
" 29	55	75	90	1,330				1,180	0	0	Negative.	
" 30	55	74	90	1,326			0.190	1,290	0.3	3.3	"	
" 31	50	76	90	1,314	49.7			1,200	0	0	"	
Sept. 1	50	76	90	1,314				1,240	0	0	"	
" 2	50	75	90	1,310				1,100	0	0	"	
" 3	50	75	90	1,310				1,000	Trace.		"	
" 4	50	75	90	1,310	49.3			1,580	0	0	"	
" 5	50	75	90	1,310				1,340	0	0	"	
" 6	50	75	90	1,310				1,100	0	0	"	
" 7	50	75	90	1,310	49.4			1,100	0	0	"	
" 8	50	75	90	1,310				1,250	0	0	"	

Under "acetone" the results from the determinations of acetone plus acetoacetic acid are listed.

Results of the determinations on the acetone bodies are expressed in terms of acetone.

Results of tests with sodium nitroprusside and ammonia are given for specimens on which quantitative determinations of the acetone bodies were not made.

TABLE III.
Case 3.

Date.	Diet.					Alveolar CO₂.	Blood sugar.	Urine.				
	Carbohydrate.	Protein.	Fat.	Calories.	Weight.			Volume.	Sugar.		Acetone.	β-hydroxybutyric acid
1921	gm.	gm.	gm.		kg.	mm.	per cent	cc.	per cent	gm.	gm.	gm
June 28							0.295	920	6.3	57.9	Positive.	
" 29	50	85	90	1,350	67.4			1,050	2.8	29.0	"	
" 30	50	85	90	1,350				1,340	1.1	14.7	"	
July 1	52	88	88	1,352				1,300	1.0	13.0	"	
" 2	25	42	45	673				1,600	0.3	4.8	"	
" 3	25	42	45	673	67.2			1,100	0	0	"	
" 4	25	42	45	673				700	0	0	"	
" 5	30	50	50	770				980	0	0	"	
" 6	30	50	55	815	67.2	36		650	0	0	"	
" 7	30	55	60	880			0.165	1,220	0	0	0.500	1.76
" 8	35	65	65	985				720	0	0	Positive.	
" 9	35	65	70	1,050				460	0	0	0.525	1.12
" 10	41	66	71	1,067	66.8	30		1,040	0	0	0.126	0.171
" 11	55	70	80	1,220		30		750	0	0	0.129	0.207
" 12	102	58	19	811		31		1,300	0	0	0.126	0.117
" 13	102	58	0	640	66.6	36		900	0	0	0.028	0.022
" 14	86	56	0	568		36		875	0	0		
" 15	80	71	19	775	66.1	33		1,450	0	0	Negative.	
" 16	81	75	29	885				1,100	0	0	0.027	0.076
" 17	85	75	40	980		37		1,700	0	0	Negative.	
" 18	85	76	40	1,004				1,775	0	0	0.085	0.066
" 19	85	74	40	996				1,860	0	0	0.059	0.049
" 20	94	74	50	1,106	65.0		0.140	1,720	0	0	0.116	0.059
" 21	90	81	60	1,224				900	0	0	0.040	0.033
" 22	90	80	70	1,314				1,700	0	0	0.042	0.057
" 23	90	76	73	1,321		38		850	0	0	0.028	0.042
" 24	95	79	89	1,487	65.8			1,820	0	0	Negative.	
" 25	95	79	100	1,596				1,170	0	0	0.128	0.133
" 26	100	84	110	1,726				1,500	0	0	0.158	0.193
" 27	100	85	110	1,730	65.7		0.130	720	0	0	0.066	0.055
" 28	100	85	110	1,730				1,180	0	0	0.107	0.333
" 29	100	84	110	1,726				1,780	0	0	0.149	0.153
" 30	100	84	110	1,726				1,300	0	0	0.032	0.038
" 31	110	92	124	1,924	65.8			1,800	0	0	Negative.	
Aug. 1	110	90	125	1,925				1,000	0	0	0.003	0.043
" 2	110	90	125	1,925				990	0	0	0.015	0.030
" 3	110	74	115	1,771	65.5			990	0	0	Negative.	

Under "acetone" the results of the determinations of acetone plus acetoacetic acid are listed.

Results of the determinations of the acetone bodies are expressed in terms of acetone.

Results of tests with sodium nitroprusside and ammonia are given on specimens on which quantitative determinations of acetone were not made.

normal amounts when diets which gave this value had been ingested for about a month. The amount of fat fed seemed to increase the excretion of acetone independently of the calculated "amount burned" as it did in the first experiment on Aug. 8, and also apparently, on Aug. 1 and 2.

Case 3 had diabetes of a mild type which had lasted only for a short time. The caloric needs of this patient appear, from a study of weight changes, to have been a little less than 120 per cent of the caloric requirement. The acetone excretion was only slightly more than normal (less than 0 1 gm. per day) when the ratio had a value of 110 per cent. The influence of increases in fat fed as distinct from total fat burned was clearly shown in this case (July 25 to 30).

CHART 3. Case 3.

Case 4 had diabetes of a mild type which was of 15 years duration. The chart shows that 120 per cent of the basal requirement represented approximately the caloric needs under the conditions of study. A ratio of glucose to fat burned, which gave a value of 90 per cent, caused an excretion of approximately 0 5 gm. of acetone, while a ratio, which gave a value of 100 per cent, caused a normal excretion. The case shows that a marked lowering of the ratio glucose to fat burned caused an increased excretion of acetone when the ratio glucose to fat fed remained unchanged. Such a relative change in the values of the ratios follows proportional reduction of the three foodstuffs.

Case 5 had a rather mild form of diabetes which had lasted for 11 years. He was confined to his bed subsequent to an operation for gangrene when the study presented here was made. His weight stayed practically con-

TABLE IV.
Case 4.

Date.	Diet.				Weight.	Alveolar CO₂.	Blood sugar.	Urine.				
	Carbohydrate.	Protein.	Fat.	Calories.				Volume.	Sugar.		Acetone.	β-hydroxybutyric acid.
1921	gm.	gm.	gm.	⁗	kg.	mm	per cent	cc.	per cent	gm.	gm.	gm.
Aug. 7	?	?	?	?	56 0			1,260	0	0	Positive.	
" 8	46	88	71	1,175				1,090	0	0	"	
" 9	45	83	90	1,322								
" 10	45	85	90	1,330				1,325	0	0	Negative.	
" 11	45	86	90	1,334	55 5			1,180	0	0	Positive.	
" 12	45	86	90	1,334			0 200	1,340	0	0	Negative.	
" 13	45	86	100	1,424				1,350	0	0	Positive.	
" 14	45	87	109	1,509	55 4			1,460	0	0	"	
" 15	48	87	129	1,701				1,470	0	0	0 366	0 190
" 16	45	86	110	1,514				1,440	0	0	0 141	0 141
" 17	45	85	110	1,510	55 7			1,320	0	0	0 187	0 246
" 18	45	89	110	1,530				1,530	0	0	0 193	0 226
" 19	45	85	110	1,510				1,510	0	0	0 236	0 378
" 20	45	85	110	1,510				1,440	0	0	0 144	0 396
" 21	50	85	109	1,521	56 2	34		1,360	0	0	Positive.	
" 22	50	85	110	1,530				1,400	0	0	0 199	0 165
" 23	50	85	110	1,530				1,420	0	0	0 130	0 085
" 24	50	85	110	1,530	56 0			1,360	0	0	0 103	0 093
" 25	50	85	111	1,539				1,740	0	0	Negative.	
" 26	50	85	110	1,530				1,460	0	0	"	
" 27	55	85	105	1,505				1,650	0	0	"	
" 28	60	85	100	1,480	56 0			1,560	0	0	"	
" 29	60	85	100	1,480				1,400	0	0	"	
" 30	60	86	100	1,484			0 210	1,200	0	0	"	
" 31	59	87	100	1,484	56 3			810	0	0	"	
Sept. 1	60	87	100	1,488				1,340	0	0	"	
" 2	60	85	100	1,480				580	0	0	"	
" 3	60	85	100	1,480				980	0	0	"	
" 4	60	84	100	1,476	55 1			1,030	0	0	"	
" 5	60	85	100	1,480				1,090	0	0		
" 6	60	85	100	1,480				1,740	0	0	Negative.	
" 7	60	86	100	1,484	55 6			1,720	0	0	"	
" 8	60	84	100	1,476				1,340	0	0	"	
" 9	60	85	100	1,480				1,530	0	0	"	
" 10	37	55	67	971				1,240	0	0	Positive.	

TABLE IV—*Concluded.*

Date.	Diet.				Weight.	Alveolar CO₂.	Blood sugar.	Urine.				β-hydroxybu-tyric acid.
	Carbohydrate	Protein.	Fat.	Calories.				Volume.	Sugar.		Acetone.	
1921	gm	gm.	gm		kg.	mm	per cent	cc.	per cent	gm.	gm.	gm.
Sept. 11	60	73	100	1,432	56 7			1,900	0	0	Negative.	
" 12	60	85	100	1,480				1,500	0	0	"	
" 13	60	84	100	1,476				1,620	0	0	"	
" 14	60	85	100	1,480	55.7			1,740	0	0	"	
" 15	60	86	100	1,484				1,930	0	0	"	
" 16	60	86	100	1,484				1,900	0	0	"	
" 17	60	86	100	1,484				1,600	0	0	"	
" 18	60	85	100	1,480	55 7			1,460	0	0	"	
" 19	60	85	100	1,480								

Under "acetone" the results from the determinations of acetone plus acetoacetic acid are listed.

Results of the determinations on the acetone bodies are expressed in terms of acetone.

Results of the test with sodium nitroprusside and ammonia are given on specimens on which quantitative determinations of the acetone bodies were not made.

CHART 4. Case 4.

TABLE V.

Case 5.

Date.	Diet.				Weight.	Alveolar CO₂.	Blood sugar.	Urine.				
	Carbohydrate.	Protein.	Fat.	Calories.				Volume.	Sugar.		Acetone.	β-hydroxybutyric acid.
1921	gm.	gm.	gm.	gm.	kg.	mm.	per cent	cc.	per cent	gm.	gm.	gm.
July 1	30	70	70	1,030				2,150	0	0	Positive.	
" 2	30	70	70	1,030				1,340	0	0	"	
" 3	30	70	70	1,030	45.2			880	0	0	"	
" 4	30	70	70	1,030				750	0	0	Negative.	
" 5	30	70	70	1,030				1,000	0	0	Positive.	
" 6	30	70	80	1,120	45.6			600	0	0	"	
" 7	30	70	80	1,120			0.170	850	0	0	0.372	0.390
" 8	30	70	80	1,120				960	0	0	Positive.	
" 9	30	71	80	1,124		37		910	0	0	0.209	0.194
" 10	30	70	87	1,183	45.8			1,000	0	0	Positive.	
" 11	30	70	80	1,120				1,130	0	0	0.133	0.076
" 12	30	70	80	1,120				1,375	0	0	Positive.	0.146
" 13	30	70	80	1,120				945	0	0	"	0.087
" 14	29	71	80	1,120				580	0	0	"	
" 15	29	70	80	1,116				725	0	0	0.256	0.130
" 16	30	70	80	1,120	45.6			1,540	0	0	0.308	0.181
" 17	30	70	80	1,120		36	0.150	1,480	0	0	Positive.	
" 18	35	69	79	1,127				1,150	0	0	0.096	0.090
" 19	35	69	80	1,136				1,590	0	0	0.119	0.062
" 20	35	69	80	1,136	45.3			1,330	0	0	0.244	0.183
" 21	35	76	80	1,164				1,360	0	0	0.173	0.294
" 22	35	75	85	1,205			0.110	1,800	0	0	0.225	0.159
" 23	35	75	85	1,201				920	0	0	Positive.	
" 24	35	75	85	1,205	46.6	31		1,640	0	0	"	
" 25	35	75	85	1,205				1,130	0	0	Negative.	
" 26	35	75	85	1,205				1,020	0	0	0.322	0.238
" 27	35	75	85	1,205	45.6							

Under "acetone" the results of determinations of acetone plus aceto-acetic acid are listed.

Results of the determinations of the acetone bodies are expressed in terms of acetone.

Results of tests with sodium nitroprusside and ammonia are given for specimens on which quantitative determinations of the acetone bodies were not made.

stant on an intake of food which furnished a smaller number of calories than would have formed the basal requirement of a normal subject of the same height and weight. The acetone excretion found in this case was fairly constant throughout the study, and was rather lower than was to be expected from the value of the ratios—60 per cent—calculated from the diet. It seems probable from a study of weight changes that in this case 120 per cent of the calculated basal requirement represents more calories than the patient actually burned in a day. He was the only patient in the series who was confined to his bed, and the only patient who maintained his body weight on such a low food intake. If the calculation of the ratio glucose to fat burned had been based on the diet which maintained weight instead of arbitrarily on 120 per cent of his calculated basal requirement, the value of the ratio which corresponded to an excretion of 0.5 gm. of acetone would have been 70 to 75 per cent. There was no reason to believe that this patient did not follow the diet, but such a possibility cannot be absolutely excluded.

CHART 5. Case 5.

Case 6 was a patient who had had diabetes for 14 years, and when he was studied his condition was rather severe. The patient received less food than would furnish calories equivalent to the basal requirement of a normal subject of the same height and weight, and showed a progressive loss of weight during the period. Increased amounts of acetone were found in almost all the specimens of urine analyzed, but the amounts were small, as compared with those expected from the numerical values of the ratio. Two analyses of the nitrogen content of the urine on the 7th and the 27th of July by the method of Folin and Denis (1916) (slightly modified to permit the use of the oxidizing and Nessler's reagent described by Folin and Wu (1919)) showed that the patient was approximately in nitrogen equilibrium on these dates. The subject was not confined to his bed, and there is no reason for believing that his caloric requirements during the day were less than those of the first four cases discussed. No

TABLE VI.

Case 6.

Date.	Diet.				Weight.	Alveolar CO₂.	Blood sugar.	Urine.			β-hydroxybutyric acid
	Carbohydrate.	Protein.	Fat.	Calories.				Volume.	Sugar.	Acetone.	
1921	gm.	gm.	gm		kg.	mm	per cent	cc.	per cent	gm.	gm.
June 23	?	?	?	?			?		3 0		Positive.
" 24	102	58	0	640	57 0		0 330	1,200	2 3	27 6	"
" 25	50	75	70	1,130				1,740	0 7	12 0	"
" 26	25	38	35	567	55 8			1,750	0 2	4 4	Negative.
" 27	33	50	47	755				1,380	Trace.	3 4	Positive.
" 28	33	50	47	755				1,580	"		"
" 29	33	50	47	755	55 6			1,830	0	0	"
" 30	26	69	50	830				1,820	0	0	Negative.
July 1	25	70	48	812				1,750	0	0	Positive.
" 2	30	70	60	940			0 225	1,860	0	0	"
" 3	30	70	70	1,030	54 7			1,260	0	0	"
" 4	30	70	70	1,030				1,240	0	0	"
" 5	35	70	75	1,095				1,540	0	0	Negative.
" 6	35	71	80	1,144	55.4			1,140	0	0	"
" 7	35	70	85	1,185				1,000	0	0	0.238	0 187
" 8	37	70	85	1,185			0 230	950	0	0	Negative.
" 9	18	36	43	603		30		910	0	0	0 090	0 106
" 10	31	40	38	626	54.3			1,240	0	0	Positive.
" 11	20	44	50	706				1,480	0	0	0 075	0 148
" 12	20	46	50	714			0 200	1,450	0	0	Positive.
" 13	21	55	55	799	54.3			1,500	0	0	0.382	0 890
" 14	20	60	55	810				945	0	0	Positive.
" 15	25	59	54	822		30		1,200	0	0	0.221	0 342
" 16	30	66	54	870				1,620	0	0	0.238	0.264
" 17	34	69	60	952		30		1,560	0	0	Positive.
" 18	35	69	60	956				1,270	0	0	0.159	0 306
" 19	35	74	60	1,020			0 200	1,300	0	0	0 130	0 076
" 20	35	75	74	1,106	53.3			1,700	0	0	0 137	0 102
" 21	35	80	79	1,171				1,330	0	0	0 114	0 082
" 22	35	80	80	1,180		33		1,310	0	0	0 130	0 127
" 23	35	80	80	1,180				1,400	0	0	Positive.
" 24	35	80	80	1,180	53.3	34		1,140	0	0	"
" 25	35	79	80	1,176				1,330	0	0	"
" 26	35	79	80	1,176				1,330	0	0	0 124	0 162
" 27	35	80	80	1,180	52.4		0 200	960	0	0	0 077	0 117

Under "acetone" the results of determinations of acetone plus aceto-acetic acid are listed.

Results of the determinations of the acetone bodies are expressed in terms of acetone.

Results of tests with sodium nitroprusside and ammonia are given for specimens on which quantitative determinations of acetone were not made.

adequate explanation can be offered for the low acetone excretion found in studying this patient.

CHART 6. Case 6.

CHART 7. Case 7.

Case 7 had a very mild case of diabetes of short duration. The food provided furnished 115 per cent more calories than her calculated requirement, and her weight was constant, but the period of observation was too

short to prove this requirement sufficient. The most striking feature shown by this case is a slight return of acetonuria—demonstrated both by quantitative and qualitative tests—when the ratio of glucose to fat burned passed from a value of 200 per cent to one of 170 per cent. This appearance of increased amounts of acetone when the ratio has such a high

TABLE VII.

Case 7.

	Diet.							Urine.				
Date.	Carbohydrate.	Protein.	Fat.	Calories.	Weight.	Alveolar CO$_2$.	Blood sugar.	Volume.	Sugar per cent	Sugar gm.	Acetone.	β-hydroxybutyric acid.
1921	gm.	gm.	gm.		kg.	mm.	per cent	cc.	per cent	gm.	gm.	gm.
Aug. 7	?	?	?	?	44.6	37		?	6 6	?	Positive.	
" 8	0	0	0	0		31		2,300	0	0	0.211	0.498
" 9	45	24	0	266		29	0.140	2,730	0	0	0.506	1.015
" 10	64	33	0	388				1,970	0	0	0.754	0.812
" 11	102	58	0	640				1,800	0	0	0.165	0.247
" 12	102	58	0	640		34		2,050	0	0	0.051	0.059
" 13	100	60	30	910				1,820	0	0	Negative.	
" 14	100	60	40	1,000	44.0			1,820	0	0	"	
" 15	100	60	50	1,090		35		1,940	0	0	"	
" 16	101	60	60	1,184				2,300	0	0	"	
" 17	100	60	80	1,360	44.3			1,980	0	0	"	
" 18	100	61	80	1,364			0.175	1,300	0	0	0.019	0.018
" 19	100	60	80	1,360		36		1,500	0	0	Negative.	
" 20	80	60	90	1,374				1,440	0	0	"	
" 21	80	61	90	1,374	45.0			1,730	0	0	Positive.	
" 22	80	61	90	1,374				1,420	0	0	0.021	0.025
" 23	80	61	90	1,374				1,430	0	0	0.108	0.081

Under "acetone" the results of the determinations of acetone plus aceto-acetic acid are listed.

Results of the determinations of the acetone bodies are expressed in terms of acetone.

Results of the test with sodium nitroprusside and ammonia are given for specimens on which quantitative analyses for the acetone bodies were not made.

value forms a marked contrast to the results obtained in the case just described, in which only small amounts of acetone were found when the ratio had a value of 60 per cent. The results in this case are similar to those found in studying one of the normal subjects as reported in a previous paper (Hubbard and Wright, 1922).

DISCUSSION.

A comparison of the acetone excretion with the molecular ratio used to express the ketogenic balance of the diets ingested shows that, in general, the excretion varied inversely with the value of the ratio based on the probable amount of fat burned by the patient during 24 hours. The amounts of acetone found were somewhat smaller than those which were found in the study of normal patients who were receiving diets which gave ratios of the same numerical values, but the border-line diet which caused a very slight increase in acetone excretion lay approximately at a value of 80 per cent, as it did in the earlier experiments. Traces of acetone were found when diets having a higher ratio were fed— as traces were occasionally found under similar conditions in some of the experiments run on normal subjects; in one of the patients studied such traces were found when the ratio had a value of 170 per cent. It seems reasonable to attribute such findings, as was done in the earlier paper, to temporary excess of ketogenic material which may have lasted for only a comparatively small part of the 24 hour period, or to a local excess of such material due to variations in the blood and nutriment supplied to different parts of the organism. One patient showed an excretion of acetone which was lower than was expected from theoretical considerations or from a comparison with other cases in the series; the tolerance of this patient for glucose was so low that it was not possible to investigate the diet which would cause no excretion of acetone.

The excretion of acetone could be largely explained by a study of the molecular ratio based on the amount of fat probably burned by the patient, but increases were sometimes found which could not be accounted for in this way. An increase in fat fed was followed by an increased excretion of acetone in some cases when this was the only change in the diet, and the additional fat fed theoretically replaced fat which the subject had been drawing from his own reserves. Such a change in the diet was shown in the numerical method used for expressing the diets, by a decrease in the value of the ratio based upon the amount of fat fed while the ratio based upon the probable amount of fat burned was not changed; in these instances the fed fat replaced only a part of the total fat

burned—a condition in which the values of the ratio based upon
the amount of total fat burned are lower than those based upon
the amount of fat fed.   The increases in acetone excretion under
these conditions were not large, and did not seem to last long.   If
the organism had not been at first able to burn completely the
large amounts of fat received at intervals with the meals, but had
later acquired the ability to do so, results similar to those found
would have been expected, and some such explanation may ac-
count for the temporary increases in acetone excretion noted.
This appearance of increased amounts of acetone with increased
intake of fat, even when the fat probably only served to replace
fat withdrawn from the body reserves, made it seem inadvisable
to furnish more fat than that which corresponds with Woodyatt's
(1921) formula for the border-line diet

$$2 \times \text{carbohydrate} + \tfrac{1}{2} \text{ protein} = \text{fat}$$

except in cases in which such increases were necessary to main-
tain life.

CONCLUSIONS.

A method has been described for calculating a molecular ratio
between ketogenic and antiketogenic compounds contained in the
diet which is applicable to diabetic patients, and seven cases have
been described in which a comparison was made between the
values of this ratio and the excretion of the acetone bodies in the
urine; it has been shown that the acetone excretion varies inversely
with the numerical values of this ratio; a study of the numerical
values of the ratio calculated for diets which correspond with a
slightly increased excretion of acetone shows that they were ap-
proximately the same as those values found for normal subjects
receiving diets low in carbohydrate, but containing sufficient
calories to supply the needs of the subject; it has been shown that
fat fed sometimes increases the amount of acetone excreted, even
when the increase replaces a part of the fat which the body was
probably withdrawing from its own reserve supplies of this
material.

BIBLIOGRAPHY.

Allen, F. M., Studies concerning glycosuria and diabetes, Cambridge, 1913.

Allen, F. M., Stillman, E., and Fitz, R., Total dietary regulation in the treatment of diabetes, New York, 1919.

Benedict, S. R., *J. Biol. Chem.*, 1918, xxxiv, 203.

Fridericia, L. S., *Hospitalstid.*, 1914, vii, 585.

Folin, O., and Denis, W., *J. Biol. Chem.*, 1916, xxvi, 473.

Folin, O., and Wu, H., *J. Biol. Chem.*, 1919, xxxviii, 81.

Hubbard, R. S., *J. Biol. Chem.*, 1921, xlix, 357.

Hubbard, R. S., and Wright, F. R., *J. Biol. Chem.*, 1922, l, 361.

Hubbard, R. S., Nicholson, S. T., Jr., and Wright, F. R., *J. Biol. Chem.*, 1922, l, p. xxviii.

Joslin, E. P., The treatment of diabetes mellitus, Philadelphia and New York, 2nd edition, 1917.

Labbé, M., Labbé, H., and Nepveux, *Compt. rend. Soc. biol.*, 1921, lxxxiv, 254.

Ladd, W. S., and Palmer, W. W., *Proc. Soc. Exp. Biol. and Med.*, 1920–21, xviii, 109.

Lang, R. M., *Biochem. J.*, 1915, ix, 456.

Lewis, R. C., and Benedict, S. R., *J. Biol. Chem.*, 1915, xx, 61.

Lusk, G., The elements of the science of nutrition, Philadelphia and London, 3rd edition, 1917.

Poulton, E. P., *Brit. Med. J.*, 1915, ii, 392.

Shaffer, P. A., *J. Biol. Chem.*, 1921, xlvii, 449.

Shaffer, P. A., *J. Biol. Chem.*, 1922, l, p. xxvi.

Woodyatt, R. T., *Arch. Int. Med.*, 1921, xxviii, 125.

Zeller, H., *Arch. Physiol.*, 1914, 213.

THE PROTEINS OF THE LIMA BEAN, PHASEOLUS LUNATUS.

By D. B. JONES, C. E. F. GERSDORFF, C. O. JOHNS, AND A. J. FINKS.

(From the Protein Investigation Laboratory, Bureau of Chemistry, United States Department of Agriculture, Washington.)

In continuation of the comparative studies of the proteins of various beans, particularly those of the genus *Phaseolus*, a study of the proteins of the lima bean is described in this paper. As far as the authors are aware, the only previous reference to any work done on this bean is a statement by Osborne (1) to the effect that some preliminary analyses which he made indicated that the chief protein constituent of this bean is the same as phaseolin obtained from the navy bean.

The results of the work described in the present study show that the proteins of the lima bean are in general quite similar to those of the other beans which have been studied: namely, the navy, *Phaseolus vulgaris*;[1] mung, *Phaseolus aureus* (2); Chinese velvet, *Stizolobium niveum* (3); Georgia velvet, *Stizolobium deeringianum* (4); aduski, *Phaseolus angularis* (5); and the jack bean, *Canavalia ensiformis* (6). Like these, the lima bean contains two globulins which differ characteristically in both sulfur and nitrogen contents. Feeding experiments with albino rats have also shown (7) a similarity in their nutritive properties. The lima bean is also deficient in cystine, and its proteins are characterized by a form of indigestibility which is remedied by cooking. On the other hand, the proteins of the lima bean differ in certain respects from those of the other beans studied, the most striking difference being the low nitrogen content of the β-globulin. The average of closely agreeing results for seven different preparations of this protein showed it to contain 14.81 per cent of nitrogen (Table III). The

[1] Studies on the proteins of the navy bean are nearly completed and the results will be published later.

231

proteins of this bean are also marked by a rather high degree of solubility in saline solutions. The soluble salts occurring naturally in the seeds are evidently sufficient to dissolve 15.13 per cent of protein when the meal is extracted with distilled water in the proportion of 2.5 cc. per gram of meal. This is practically as much as was extracted by a 3 per cent sodium chloride solution (Table I).

The meal used for the preparation of the proteins described in this paper was obtained from beans of two varieties, the "Fordhook bush" and "Carpenteria pole," which were obtained in the open market. No differences were observed in the results obtained from these two varieties. The meal contained 21.17 per cent of protein ($N \times 6.25$). 3 per cent sodium chloride solution at room temperature extracted 72.32 per cent of the total protein in the meal, or 15.31 per cent based on the amount of meal used.

The α- and β-globulins were separated by fractional precipitation from 3 per cent sodium chloride solution by means of ammonium sulfate. The α-globulin was precipitated by addition of ammonium sulfate until the solution was brought up to 0.25 of saturation (19.07 gm. of ammonium sulfate per 100 cc. of the final solution). The β-globulin separated between 0.45 and 0.75 of saturation.

An albumin, amounting to 1.75 per cent of the meal, or 8.26 per cent of the total protein, was obtained by boiling a distilled water extract of the meal after all of the globulins had been removed. This percentage of albumin is considerably higher than that obtained from the other beans which have been studied.

The percentages of the basic amino-acids in the three proteins isolated were determined by Van Slyke's method, with results as given in Tables VI, VIII, and X.

EXPERIMENTAL.

Preliminary Experiments.—Extraction experiments were made with the bean meal, using various concentrations of sodium chloride in water in the proportion of 2.5 cc. of solvent to each gram of meal. The mixtures were allowed to stand for 3 hours at room temperature, with shaking at intervals of about 15 minutes. Nitrogen determinations made on the filtered extracts showed that the maximum amount of protein was extracted by a 3 per cent solution of sodium chloride (Table I).

Preparation of the α-Globulin.—For each preparation of the α-globulin about 3.5 kilos of meal were extracted with 3 per cent sodium chloride solution. Filter paper scraps were then pulped in the mixtures until a consistency suitable for pressing was obtained, and the mixtures were pressed in muslin bags. The expressed liquors were filtered clear by suction through a mat of paper pulp, and the filtrates made 0.25 saturated by addition of solid ammonium sulfate. After standing over night the precipitates were collected on folded filter papers and washed with 3 per cent sodium chloride solution which had been previously made 0.25 saturated with ammonium sulfate. Since these precipitates required very large volumes of the sodium chloride solution to redissolve them, they were dialyzed in suspension in this solvent against chilled running water for 11 days. The proteins which had separated were washed with distilled water until free from chlorides and sulfates, and dried with alcohol and ether in

TABLE I.

Extraction Experiments. *

Solvent.	Protein extract from meal ($N \times 6.25$).
	per cent
Distilled water..	15.13
1.0 per cent NaCl....................................	15.19
3.0 " " "	15.31
8.0 " " "	14.50
10.0 " " "	14.06
15.0 " " "	13.63
20.0 " " "	11.84

* The extractions were carried out at room temperature, for a period of 3 hours each, with frequent stirring. Solvents were used in the proportion of 2.5 cc. per gm. of meal.

the usual way. An average yield of 2.74 per cent of the total protein, or 0.58 per cent of the meal used, was obtained. Average results of duplicate analyses of five preparations of the α-globulin are given in Table II. The analyses are calculated on an ash- and moisture-free basis.

Preparation of the β-Globulin.—A small intermediate fraction consisting of a mixture of the α- and β-fractions was removed and discarded. This fraction was obtained by bringing the filtrates from the original precipitates of the α-globulin, which were already 0.25 saturated, up to 0.4 of saturation with ammonium sulfate. The filtrates from the intermediate fraction were then made 0.75 saturated by addition of more ammonium sulfate, and the precipitated β-globulin was filtered and washed in the manner described in the case of the α-globulin. The precipitates were then redissolved in the minimum amount of distilled water and dialyzed for 13 days. An average yield of 1.58 per cent of the meal extracted was obtained.

Seven preparations were made which gave closely agreeing results on elementary analyses. Average results of duplicate analyses are given in Table III.

TABLE II.

*Average Results of Duplicate Analyses of the α-Globulin.**

	Preparation.					
	I	II	III	IV	V	Average.
	per cent	per cent	per cent	per cent	per cent	per cent
C.............	53 42	53.66	53.69	53.63	53.87	53.65
H.............	6.88	6.60	6.59	6.74	6.45	6.65
N.............	15.84	15.57	15.56	15.28	15.50	15.55
S.............	1.29	1.23	1.27	1.28	1.30	1.27
O.............	22.57	22.94	22.89	23.07	22.88	22.88
Moisture.......	5.44	7.52	7.25	7.36	7.77	
Ash...........	1.52	0.44	0.65	0.43	0.57	

* Calculated on an ash- and moisture-free basis.

TABLE III.

*Average Results of Duplicate Analyses of the β-Globulin.**

	Preparation.							
	I	II	III	IV	V	VI	VII	Aver-age.
	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent
C.............	52.59	52.61	52.80	52.70	52.78	52.72	52.85	52.72
H.............	6.61	6.78	6.83	6.83	6.74	6.74	6.83	6.77
N.............	14.89	14.90	14.85	14.60	14.82	14.80	14.85	14.81
S.............	0.37	0.33	0.35	0.36	0.37	0.34	0.36	0.35
O.............	25.54	25.38	25.17	25.51	25.29	25.40	25.11	25.35
Moisture.......	8.76	6.96	9.27	5.66	6.58	7.99	6.76	
Ash...........	0.80	1.08	0.61	0.49	0.44	0.71	0.69	

* Calculated on an ash- and moisture-free basis.

Properties of the Globulins.—Both globulins, when prepared and dried as described, consisted of dusty powders. The α-globulin had a deep cream color, while the β-globulin was pure white. The two globulins are fairly well differentiated by their precipitation limits with ammonium sulfate. Precipitation of the α-globulin began at 0.15 of saturation and became flocculent at 0.25 of

saturation.   Between 0.3 and 0.4 of saturation a small precipitate
was obtained, which had a sulfur content of 0.63 per cent, indicat-
ing that it was a mixture of the two globulins.   The β-globulin
precipitated at 0.45 to 0.75 of saturation.

Coagulation temperatures of the globulins, determined on their
saline extracts which had been slightly acidified with dilute
acetic acid, showed that the α-globulin coagulated at about 68° C,
and the β-globulin at about 95° C.

Both globulins gave positive tests for tryptophane with the
Hopkins and Cole reagent (8), the color developing immediately
in the case of the β-globulin, while the α-globulin required more
time for the color development.

TABLE IV.

*Average Results of Duplicate Analyses of the Albumin.**

	Preparation.		
	I	II	Average.
	per cent	per cent	per cent
C	54.14	54.19	54.17
H	6.62	6.64	6.63
N	14.20	14.24	14.22
S	1.15	1.15	1.15
O	23.89	23.78	23.83
Moisture	9.21	8.72	
Ash	1.68	3.19	

* Calculated on an ash- and moisture-free basis.

The Albumin.—2 liters of water and 500 gm. of meal were mixed and
passed three times through a peanut grinder. The mixture was then
pressed, filtered in the usual way, and the clear extract dialyzed for 5 days.
The precipitated globulins were removed by filtration, and the filtrate was
again dialyzed for 8 days. After removing a small amount of precipitated
globulin, the solution was saturated with carbon dioxide which caused the
further separation of a small amount of precipitate. This substance was
filtered and the albumin was coagulated by boiling the filtrate which had
been previously slightly acidified with acetic acid. After washing with hot
water and drying in the usual way at 55°C., the albumin consisted of a
cream white amorphous powder. The yield amounted to 1.75 per cent
of the meal. The average results of duplicate analyses of two preparations
are given in Table IV. The albumin gave a faint, though decided, test for
tryptophane.

Analyses of the α- and β-Globulins and of the Albumin by the Van Slyke Method.—Duplicate samples of 3 gm. each of the globulins and albumin were hydrolyzed by boiling for about 30 hours with

TABLE V.

*Distribution of Nitrogen in the α-Globulin as Determined by the Van Slyke Method.**

Sample I, ash- and moisture-free, 2.7630 gm. protein, 0.4299 gm. nitrogen.†
" II, " " " " 2.7630 " " 0.4299 " "

	Preparation.				
	I	II	I	II	Average.
	gm.	gm.	per cent	per cent	per cent
Amide N	0.0442	0.0444	10.28	10.33	10.31
Humin N adsorbed by lime	0.0086	0.0087	2.00	2.02	2.01
Humin N in amyl alcohol-ether extract	0.0008	0.0010	0.19	0.23	0.21
Cystine N	0.0052	0.0051	1.21	1.19	1.20
Arginine N	0.0506	0.0502	11.77	11.68	11.72
Histidine N	0.0276	0.0279	6.42	6.49	6.46
Lysine N	0.0418	0.0413	9.72	9.61	9.67
Amino N of filtrate	0.2389	0.2400	55.57	55.82	55.69
Non-amino N of filtrate	0.0127	0.0122	2.95	2.84	2.89
Total N regained	0.4304	0.4308	100.11	100.21	100.16

* Nitrogen figures corrected for the solubility of the bases.
† Nitrogen content of protein, 15.56 per cent.

TABLE VI.

Basic Amino-Acids in the α-Globulin.

Amino-acid.	I	II	Average.
	per cent	per cent	per cent
Cystine	1.61	1.58	1.60
Arginine	5.69	5.65	5.67
Histidine	3.69	3.73	3.71
Lysine	7.89	7.80	7.84
Tryptophane			Present.

100 cc. of 20 per cent hydrochloric acid. The phosphotungstates of the bases were decomposed by the amyl alcohol-ether method (9). The results of the analyses are given in Tables V to XI.

TABLE VII.

Distribution of Nitrogen in the β-Globulin as Determined by the Van Slyke Method.

Sample I, ash- and moisture-free, 2.7390 gm. protein, 0.4054 gm. nitrogen.†
"   II,   "   "   "   "   2.7390 "   "   0.4054 "   "

	Preparation.				
	I	II	I	II	Average.
	gm.	gm.	per cent	per cent	per cent
Amide N	0.0414	0.0412	10.21	10.17	10.19
Humin N adsorbed by lime	0.0062	0.0065	1.54	1.60	1.57
Humin N in amyl alcohol-ether extract	0.0003	0.0003	0.07	0.07	0.07
Cystine N	0.0027	0.0027	0.67	0.67	0.67
Arginine N	0.0443	0.0450	10.93	11.10	11.02
Histidine N	0.0199	0.0190	4.91	4.69	4.80
Lysine N	0.0450	0.0445	11.10	10.98	11.04
Amino N of filtrate	0.2422	0.2422	59.75	59.75	59.75
Non-amino N of filtrate	0.0060	0.0066	1.48	1.63	1.55
Total N regained	0.4080	0.4080	100.66	100.66	100.66

* Nitrogen figures corrected for the solubility of the bases.
† Nitrogen content of protein, 14.80 per cent.

TABLE VIII.

Basic Amino-Acids in the β-Globulin.

Amino-acid.	I	II	Average.
	per cent	per cent	per cent
Cystine	0.84	0.84	0.84
Arginine	5.03	5.11	5.07
Histidine	2.68	2.56	2.62
Lysine	8.57	8.48	8.53
Tryptophane			Present.

TABLE IX.

*Distribution of Nitrogen in the Albumin as Determined by the Van Slyke Method.**

Sample 1, ash- and moisture-free, 2.6427 gm. protein, 0.3763 gm. nitrogen.†
" 11, " " " " 2.6427 " " 0.3763 " "

	Preparation.				
	I	II	I	II	Average.
	gm.	gm.	per cent	per cent	per cent
Amide N...	0.0367	0.0369	9.75	9.81	9.78
Humin N adsorbed by lime	0.0070	0.0070	1.86	1.86	1.86
Humin N in amyl alcohol-ether extract.	0.0007	0.0007	0.19	0.19	0.19
Cystine N......	0.0033	0.0033	0.88	0.88	0.88
Arginine N	0.0488	0.0488	12.97	12.97	12.97
Histidine N	0.0188	0.0179	5.00	4.76	4.88
Lysine N	0.0298	0.0307	7.92	8.16	8.04
Amino N of filtrate	0.2358	0.2358	62.66	62.66	62.66
Non-amino N of filtrate	0.0023	0.0023	0.61	0.61	0.61
Total N regained	0.3832	0.3834	101.84	101.90	101.87

* Nitrogen figures corrected for the solubility of the bases.
† Nitrogen content of protein, 14.24 per cent.

TABLE X.

Basic Amino-Acids in the Albumin.

Amino-acid.	I	II	Average.
	per cent	per cent	per cent
Cystine	1.07	1.07	1.07
Arginine	5.74	5.74	5.74
Histidine	2.63	2.44	2.54
Lysine	5.88	6.06	5.97
Tryptophane			Present.

TABLE XI.

Distribution of Nitrogen in the α- and β-Globulins, and in the Albumin as Calculated from the Van Slyke Analyses in Terms of Percentage of the Proteins.

N	α-Globulin.*			β-Globulin.†			Albumin.‡		
	I	II	Average.	I	II	Average.	I	II	Average.
	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent
Amide	1.60	1.61	1.61	1.51	1.50	1.51	1.39	1.40	1.40
Humin	0.34	0.35	0.34	0.24	0.25	0.24	0.29	0.29	0.29
Basic	4.53	4.51	4.52	4.09	4.06	4.08	3.81	3.81	3.81
Non-basic	9.11	9.13	9.12	9.06	9.08	9.07	9.01	9.01	9.01
Total	15.58	15.60	15.59	14.90	14.89	14.90	14.50	14.51	14.51

* Nitrogen content, 15.56 per cent.

† Nitrogen content, 14.80 per cent.

‡ Nitrogen content, 14.24 per cent.

SUMMARY.

The lima bean meal used for the extraction of the proteins contained 21.17 per cent of protein ($N \times 6.25$). 3 per cent sodium chloride solution at room temperature extracted 72.32 per cent of the total protein, or 15.31 per cent based on the weight of the meal used.

Two globulins were isolated by fractional precipitation of the sodium chloride extracts by means of ammonium sulfate. The α-globulin was precipitated by addition of ammonium sulfate until the original extract was 0.25 saturated. The β-globulin separated between 0.45 and 0.75 of saturation. A small fraction intermediate between the α- and β-globulins was removed and discarded. This fraction consisted of a mixture of the two globulins.

An albumin, amounting to 1.75 per cent of the meal, or 8.25 per cent of the total protein, was obtained from distilled water extracts of the bean meal after the globulins had been removed.

Elementary analyses of the three proteins isolated and determination of the basic amino-acids by the Van Slyke method show in general the same differences as have been found between the corresponding proteins obtained from other beans which have been

studied. Both globulins and the albumin gave positive tests for tryptophane.

As shown in a previous publication on the nutritive value of the proteins of the lima bean, its total proteins are deficient in cystine and are characterized by a form of indigestibility which is remedied by cooking.

BIBLIOGRAPHY.

1. Osborne, T. B., *Ergebn. Physiol.*, 1910, x, 119.
2. Johns, C. O., and Waterman, H. C., *J. Biol. Chem.*, 1920, xliv, 303.
3. Johns, C. O., and Finks, A. J., *J. Biol. Chem.*, 1918, xxxiv, 429.
4. Johns, C. O., and Waterman, H. C., *J. Biol. Chem.*, 1920, xlii, 59.
5. Jones, D. B., Finks, A. J., and Gersdorff, C. E. F., *J. Biol. Chem.*, 1922, li, 103.
6. Jones, D. B., and Johns, C. O., *J. Biol. Chem.*, 1916–17, xxviii, 67.
7. Finks, A. J., and Johns, C. O., *Am. J. Physiol.*, 1921, lvi, 205.
8. Hopkins, F. G., and Cole, S. W., *J. Physiol.*, 1901–02, xxvii, 418.
9. Van Slyke, D. D., *J. Biol. Chem.*, 1915, xxii, 281.

THE DISTRIBUTION OF SODIUM, POTASSIUM, CALCIUM, AND MAGNESIUM BETWEEN THE CORPUSCLES AND SERUM OF HUMAN BLOOD.

By BENJAMIN KRAMER and FREDERICK F. TISDALL.

(From the Department of Pediatrics, the Johns Hopkins University Baltimore.)

A knowledge of the concentration of sodium, potassium, calcium, and magnesium in the blood is necessary in connection with a variety of problems. Methods hitherto used for the quantitative determination of these elements in blood have required large amounts of material even for a single determination and have been, in most cases, difficult to carry out. These facts have tended to discourage such studies, particularly with human blood.

The earliest recorded figures for the concentration of cations and anions in human blood, corpuscles, and serum, are those reported by Schmidt (1) in 1850. Wanach (2) in 1888 estimated the sodium and potassium in the blood corpuscles and serum of eight adults. Some years before this Bunge (3), a pupil of Schmidt, reported a number of complete analyses of the ash of the blood corpuscles and serum of several animals. Abderhalden (4) later published the results of a similar study on a larger series of animals. No studies on the distribution of cations between the corpuscles and serum of normal human blood have appeared since those of Wanach.[1]

The introduction of methods for the quantitative determination of sodium, potassium, calcium, and magnesium with small amounts of serum and whole blood (5 to 9) has made such studies on human

---

[1] After this paper had been written an article appeared on the effect of changes in CO_2 tension upon the distribution of sodium, potassium, chlorine, phosphorus, and bicarbonate between the corpuscles and plasma of defibrinated and filtered beef blood (Doisy, E. A., and Eaton, E. P., *J. Biol. Chem.*, 1921, xlvii, 377).

subjects possible. We have investigated the concentration of
these elements in the venous blood and serum of normal adults.
The determinations were made on serum in preference to plasma
because the addition of an anticoagulant (usually a salt) is thereby
avoided. This permits the determination of all the cations to be
made on the same sample. Hemolysis occurs less frequently with
serum than plasma. We have found the potassium and calcium
content of citrated plasma to be practically the same as that of
serum. Schmidt (1) has shown that the inorganic composition of
plasma is identical with that of serum.

We have repeatedly demonstrated the remarkable constancy
of the concentration of the elements (sodium, potassium, calcium,

TABLE I.

Concentration of Sodium in Blood Serum and Corpuscles of Normal Adults.

Sample.	Plasma.	Na per 100 cc. serum.	Na per 100 cc. blood.	Na in plasma of 100 cc of blood.	Na in 100 cc. corpuscles calculated.
	per cent	mg.	mg.	mg.	mg.
1	59	335	193	198	−12
2	65	335	220	218	+ 6
3	58	335	195	194	+ 3
4	57	335	186	191	−12
5	58	335	187	194	−17
6	56	335	189	187	+ 5
7	60	335	199	201	− 5

and magnesium), in the serum of normal adults and have there-
fore assumed in Tables I and II the average of our previously
reported figures for sodium and potassium as representing the
concentration of these elements in the serum of the normal adult
male. The concentration of calcium was determined directly on
ashed plasma. The whole blood samples were collected in dis-
tilled water and weighed and the cations determined by methods
referred to above. The relative proportion of corpuscles to
plasma was determined by the use of hematocrit, and the concen-
tration of the various elements in the corpuscles calculated from
the assumed concentration of the respective element in the serum,
its concentration in whole blood, and the percentage of corpuscles.

The results of our determinations are given in Tables I to VI.

Column 2 of Table I shows the proportion of plasma in the blood samples analyzed; Column 3, the average value for sodium expressed in mg. per 100 cc. of serum for all our determinations with sera of normal adults.   The figures for sodium of whole blood are given in Column 4.   The number of mg. of sodium present in the amount of serum contained in 100 cc. of the given whole blood sample, is given in Column 5.   It will be seen that the latter is practically the same as the sodium concentration in the whole blood of the sample; *i.e.*, that there is no sodium in the corpuscles.

TABLE II.

Concentration of Potassium in Blood Serum and Corpuscles of Normal Adults.

Sample.	Plasma.	K per 100 cc. serum.	K per 100 cc. blood.	K per 100 cc. corpuscles.
	per cent	mg	mg.	mg
1	61	19.5	172	410
2	60	19.5	187	438
3	57	19.5	188	410
4	68	19.5	153	437
5	58	19.5	186	420
6	57	19.5	200	438
7	61	19.5	180	430
8	62	19.5	175	428
9	57	19.5	202	444
10	59	19.5	193	441
11	65	19.5	164	413
12	65	19.5	169	425
13	56	19.5	201	430
Average.....	60.5	19.5	182	428

Column 4 of Table II gives the figures for the number of mg. of potassium per 100 cc. of whole blood. We have calculated the concentration of the same element in 100 cc. of corpuscles and placed the results in Column 5. It is evident that the K content of the blood of the adult man varies directly as the corpuscular content. With a corpuscular content of 43 per cent (Sample 3) the K of 100 cc. of whole blood was 188 mg. whereas when the percentage of corpuscles dropped to 32 (Sample 4) the K content of the whole blood fell to 153 mg. per 100 cc. This is shown in a more striking manner if one calculates the K content of the corpuscles. When this is done the constancy of the K content of the

corpuscles becomes apparent (see Column 5).   Since, as we shall
see later, the corpuscles contain no calcium and only traces of
magnesium and, as we have seen from results recorded in Table I,
no sodium, it becomes obvious that potassium constitutes practi-
cally all the fixed mineral base of the corpuscles.   In this respect
man differs from a number of animals, namely the dog and cat,

TABLE III.

Concentration of Calcium in Blood Serum and Corpuscles of Normal Adults.

Sample.	Plasma.	Ca per 100 cc. plasma.	Ca per 100 cc. blood.	Calcium in plasma of 100 cc. blood	Ca in 100 cc. of corpuscles calculated.
	per cent	*mg.*	*mg.*	*mg.*	*mg.*
1	58	10.0	5.3	5.8	−1.2
2	57	9.5	5.3	5.4	−0.2
3	72	9.5	6.7	6.8	−0.4
4	59	9.8	6.2	5.8	+1.0
5	58	9.5	5.3	5.4	−0.2
6	65	9.3	5.9	6.0	−0.3
7	57	9.7	5.5	5.5	±0.0

TABLE IV.

Concentration of Magnesium in Blood and Serum of Normal Adults.

Sample.	Magnesium per 100 cc. whole blood.	Magnesium per 100 cc. serum.
	mg.	
1	2.6	
2	4.0	
3	3.8	
4	3.8	
5	2.8	
6	2.8	
7	3.8	
8	2.3	
Average.............	3.2	2.5

whose corpuscles contain potassium in practically the same con-
centration as does their plasma.   These animals make up their
deficit of potassium in the corpuscles with sodium.

   Table III shows that in seven consecutive instances, we analyzed
the calcium of the serum and whole blood and calculated from
these data and the hematocrit reading the calcium of the cor-

puscles. In not a single instance were we able to demonstrate, with certainty, the presence of calcium in the corpuscles.

Table IV requires no explanation.

SUMMARY.

1. We have previously shown that the concentrations of sodium, potassium, calcium, and magnesium in the sera of normal adults and children are singularly constant.

2. Table I shows that human corpuscles are practically free of sodium.

3. The concentration of potassium in human corpuscles is remarkably constant varying only from 410 to 440 mg. per 100 cc. of corpuscles. The average value found for thirteen samples was 428 mg. This is about twenty times the concentration of the same element in serum. Potassium represents practically all the fixed mineral base of human corpuscles.

4. Only about 2 to 4 mg. of magnesium are present in 100 cc. of corpuscles.

5. The magnesium content of whole blood varies from 2.3 to 4.0 mg. per 100 cc. These figures agree with those reported by earlier investigators.

6. In a study of seven consecutive normal bloods we found practically no calcium in the corpuscles.

The Presence of Calcium in Corpuscles.—The question of the presence of calcium in blood corpuscles has recently been the subject of considerable discussion. The earlier workers (4) regularly found no calcium in the corpuscles. More recently a number of investigators have reported the finding of considerable amounts of calcium in blood corpuscles. Hamburger (10) found as much as 32 mg. of calcium per 100 cc. of corpuscles. Rona and Takahashi (11) were able to demonstrate the presence of only 1.0 to 2.4 mg. in an equal volume of corpuscles while Heubner and Rona (12) found demonstrable amounts of calcium in the red blood cells of only six cats in a series of twenty-six animals studied. Cowie and Calhoun (13) maintain that considerable amounts of calcium are present in corpuscles and in more recent publications Jones and Nye, and Jones (14) insist upon the presence of calcium in corpuscles in appreciable amounts.

Howland and Marriott (15) found human corpuscles to be free of calcium. Lamers (16) concluded from his analyses of the blood of healthy women and of women suffering with a variety of diseases, that detectable amounts of calcium do not occur in human corpuscles. Richter-Quittner and Falta (17) also failed to find calcium either in human blood corpuscles or in those of animals. In a series of seven consecutive samples of blood from seven normal adults we have likewise been unable to demonstrate the presence of calcium in the corpuscles of a single individual. We shall not enter here into a description of the various controls which we have made to convince ourselves of the accuracy of our methods both for serum as well as for whole blood. These have been described elsewhere in detail. We are convinced, and our conviction is based upon a large number of analyses of human blood, and of the blood of a variety of animals, that the calcium of serum or plasma is remarkably constant for a large variety of normal animals (man, dog, rat, sheep, and cow), varying only from 9 to 11 mg. per 100 cc. of serum. The concentration of the same element in whole blood shows a much greater fluctuation because of the variation in the percentage of corpuscles in different samples. Nevertheless, the figure for whole blood rarely exceeds 7 mg. per 100 cc. of blood. Neither does it fall, except in cases of marked polycythemia, below 5 mg. Table III illustrates some of these statements. We have been unable to demonstrate the presence of calcium in appreciable amounts in the corpuscles of the normal adult and are inclined to attribute the finding of calcium in the corpuscles by others to errors in their calcium determinations. The sources of such errors in the determination of minute amounts of calcium have been discussed by Kramer, Tisdall, and Howland (18).

A simple example in which the figures used represent the average of a large series of determinations made by Jones and Nye (14) on the blood of normal boys and girls, will serve to illustrate the usual error in such investigations. The average calcium concentration per 100 cc. of plasma was found by them to be 10.1 mg., that of whole blood 9.4 mg., and the percentage of corpuscles was 38.4. If there were no calcium in the corpuscles the concentration of calcium per 100 cc. of whole blood should have been $10.1 \times 61.6 = 6.2$ mg. A glance at Table III shows this to be a

normal value for the calcium concentration of whole blood. The amount actually found by Jones and Nye was 9.4 mg. They used the method of Lyman (19) for their calcium determinations. It has been pointed out elsewhere (18) that when the supernatant fluid obtained after precipitating plasma proteins with trichloro-acetic acid (as performed in the Lyman procedure) is filtered through even good grades of acid-washed filter paper, calcium, in variable, but demonstrable amounts, may enter the filtrate and be responsible for many of the high values for calcium frequently obtained.

Existence of Alkali Protein Compounds in Blood.—The concentrations of chlorine and bicarbonate have been repeatedly determined for normal serum and plasma by many investigators. We have tabulated some of these results in Table V in grams, gram equivalents, and their acid equivalent expressed as cc. of 0.1 N acid per liter. The concentrations of chlorine and bicarbonate in corpuscles have been determined by Fridericia (20). Means (21) and his collaborators have also studied the distribution of bicarbonate between the corpuscles and plasma of normal adults. Some of these determinations are given in Tables V and VI. The value for the concentration of inorganic phosphorus in the serum represents the average of a large number of determinations by ourselves. We have accepted Bloor's statement (22) that the inorganic phosphorus of corpuscles is about twice that of serum.[2] De Boer (23) found the concentration of sulfate in plasma to be 0.002 M. The figures recorded for the concentration of sodium, potassium, calcium, and magnesium in corpuscles and serum are the averages of all the determinations which we have made.

If one calculates the concentrations of acid and basic equivalents found in serum and corpuscles, it is found that in each case there is an excess of base of about 16 per cent. Since the pH of normal blood is about 7.35, this base cannot be free. In considering substances that occur in normal blood and might bind fixed base one thinks of (*a*) proteins functioning as acids (Loeb, 24) and (*b*) organic acids, including lactic acid and amino-acids. In a recent review Van Slyke (25) states:

[2] This statement has recently been challenged by Zucker and Gutman, who maintained that the inorganic phosphorus concentration is the same both inside and outside the red blood cells (Zucker, T. F., and Gutman, M. B., *Proc. Soc. Exp. Biol. and Med.*, 1921–22, xix, 169).

". . . . it is practically certain that it (blood) contains no substances in considerable amount of which we do not have at least sufficient knowledge to tell whether or not they can act as buffers, i.e., whether or not they are salts of weak acids or bases. An examination of the constitutents (of blood) reveals among those present in amounts sufficient to have significant effect only the proteins, the bicarbonate, and the phosphate, which can be expected to act as CO_2 carrying buffers."

These and chlorine, therefore, would represent the base-binding substances of blood.

TABLE V.

Concentration of Basic and Acid Radicles in Serum.

	Per liter.	Gram equivalents per liter.	0 1 N base.
Basic radicles.			
	gm.		*cc.*
Na...............	3.350	0.1460	1,460
K................	0.200	0.0050	50
Ca................	0.100	0.0050	50
Mg...............	0.030	0.0025	25
Total...........		0.1585	1,585
Acid radicles.			
−Cl..............	3.600	0.1010	1,010
−HCO_3...........	1.630	0.0267	267
−HPO_4...........	0.092	0.0010*	18
−SO_4.............	0.192	0.0040	40
Total....		0.1327	1,335
Excess of base....		0.0258	250

Percentage of base not combined with acid radicles, 16 per cent.

* This figure is really the molar concentration of HPO_4 rather than the equivalent. The difference, however, is so small as to be negligible. The equivalent of 0.1 N base has been calculated on the basis of what the ratio would be for $\dfrac{N_2HPO_4}{NaH_2PO_4}$ at pH 7.35.

According to Campbell and Poulton (26) the isoelectric point of hemoglobin is at pH 6.98. Michaelis (27) gives the isoelectric point of serum globulin as pH 5.5 and that of serum albumin as pH 4.7. The pH of normal serum is 7.35 and that of corpuscles

differs probably only slightly from this figure (28). Loeb (24) has demonstrated that at a pH greater than that of their isoelectric point proteins function as acids forming readily dissociable salts with univalent cations. These facts make the existence of protein cation compounds in serum and corpuscles highly probable.

Bloor (22) has shown that the so called undetermined phosphorus in the corpuscles may be very high. This fraction he considers as possibly an organic acid. Since we know practically nothing as to its nature, it is idle to speculate as to the possibility of its

TABLE VI.

Concentration of Basic and Acid Radicles in Corpuscles.

	Per liter.	Gram equivalents per liter.	0.1 N base.
Basic radicles.			
	gm.		*cc.*
K...............	4.280	0.1097	1,097
Mg..............	0.050	0.0040	40
Total..........		0.1137	1,137
Acid radicles.			
−HCO$_3$...........	1.680	0.0276	276
−Cl.............	2.230	0.0628	628
−HPO$_4$...........	0.182	0.0020	36
Total..........		0.0924	940
Excess of base....		0.0213	197

Per cent of base not combined with acid radicles, 17 per cent.

forming any compounds with potassium. Ryffel (29) has shown that even normal blood may contain 0.012 per cent lactic acid. Nevertheless, the ease with which this acid is formed *in vitro* in biological material containing sugar raises the question of its actual existence in the circulation under normal conditions. Amino-acids might possibly bind 20 cc. of 0.1 N base in the serum and 60 cc. in the corpuscles (30).

We may conclude, therefore, that there are other substances in normal serum and corpuscles, beside the well known anions,

which bind base. These substances are probably for the most part proteins functioning as acids.

Total Available Fixed Base of Blood.

Table V shows that the sodium content of 1 liter of serum is equal to 1,460 cc. of 0.1 N base. The total available base of 1 liter of serum is equal to 1,585 cc. of 0.1 N base: i.e., sodium represents 92 per cent of the mineral base of serum. The remaining 8 per cent is represented by calcium, magnesium, and potassium. It has been shown elsewhere that the magnesium concentration of serum varies but little with normal individuals, as well as with those suffering from a variety of pathological conditions. Hence for practical purposes the concentration of this element may be considered as a constant. The concentration of calcium is likewise a fixed quantity except with nephritis in adults and tetany in children (18). Here the calcium concentration of the serum is rarely reduced below 5 mg. per 100 cc. of serum. Furthermore, the potassium concentration is usually increased under the same circumstances (31). The increase of potassium is usually compensated for wholly or in part by a decrease of the calcium concentration. A decrease of the calcium concentration to 5 mg. reduces the figure for fixed base by an amount equal to 25 cc. of 0.1 N base, while an increase of potassium to 30 mg., the maximum which we have found in any case, corresponds likewise to an increase of fixed base of 25 cc. of 0.1 N base so that if we assume the concentration of K, Ca, and Mg as unchanged and add this value expressed as 0.1 N base to the figure, expressed in similar terms, obtained by actually determining the concentration of sodium in serum we obtain, within ± 5 per cent, a measure of the total fixed base of the serum. The only element whose concentration must be determined is sodium and this can be done on 1 to 2 cc. of serum with an error that does not exceed ± 3 per cent. In a similar manner the total fixed base of corpuscles can be determined by finding the potassium concentration of whole blood, the proportion of corpuscles to serum, and assuming the K concentration of serum as 20 mg. per 100 cc.

CONCLUSIONS.

1. The corpuscles of human blood do not contain appreciable amounts of sodium or calcium.

2. The average concentration of potassium per 100 cc. of corpuscles found in thirteen normal adults was 428 mg.

3. The concentration of magnesium in whole blood is slightly higher than that of serum.

4. The extent to which the concentration of sodium, potassium, calcium, and magnesium in whole blood and corpuscles may vary is indicated in the tables.

5. Evidence is presented showing that there is an excess of about 16 per cent of basic radicles over the well known acid radicles in both serum and corpuscles. It is likely that the excess is in combination with proteins.

6. Sodium represents about 92 per cent of the fixed base of serum; potassium, practically all that of corpuscles.

BIBLIOGRAPHY.

1. Schmidt, C., Charakteristik der Epidemischen Cholera, Leipsic and Mitau, 1850, 29, 32.
2. Wanach, R., *Jahresb. Thierchem.*, 1888–89, xviii, 88.
3. Bunge, G., *Z. Biol.*, 1876, xii, 191.
4. Abderhalden, E., *Z. physiol. Chem.*, 1898, xxv, 65.
5. Kramer, B., and Tisdall, F. F., *J. Biol. Chem.*, 1921, xlvi, 467.
6. Kramer, B., and Tisdall, F. F., *J. Biol. Chem.*, 1921, xlvi, 339.
7. Kramer, B., and Tisdall, F. F., *J. Biol. Chem.*, 1921, xlvii, 475.
8. Kramer, B., and Tisdall, F. F., *Bull. Johns Hopkins Hosp.*, 1921, xxxii, 44.
9. Kramer, B., and Tisdall, F. F., *J. Biol. Chem.*, 1921, xlviii, 223.
10. Hamburger, H. J., *Z. physik. Chem.*, 1909, lxix, 663.
11. Rona, P., and Takahashi, D., *Biochem. Z.*, 1911, xxxi, 337.
12. Heubner, W., and Rona, P., *Biochem. Z.*, 1918–19, xciii, 187.
13. Cowie, D. M., and Calhoun, H. A., *J. Biol. Chem.*, 1919, xxxvii, 505.
14. Jones, M. R., and Nye, L. L., *J. Biol. Chem.*, 1921, xlvii, 321. Jones, M. R., *J. Biol. Chem.*, 1921, xlix, 187.
15. Howland, J., and Marriott, W. McK., *Quart. J. Med.*, 1917–18, xi, 289. Marriott, W. McK., and Howland, J., *J. Biol. Chem.*, 1917, xxxii, 233.
16. Lamers, A. J. M., *Z. Geburtsh. u. Gynäk.*, 1912, lxxi, 393.
17. Richter-Quittner, M., *Wien. Arch.*, 1921, ii, 217. Falta, W., *Wien. Arch.*, 1921, ii, 187.
18. Kramer, B., Tisdall, F. F., and Howland, J., *Am. J. Dis. Child.*, 1921, xxii, 560.

19. Lyman, H., *J. Biol. Chem.*, 1917, xxix, 169.
20. Fridericia, L. S., *J. Biol. Chem.*, 1920, xlii, 245.
21. Smith, L. W., Means, J. H., and Woodwell, M. N., *J. Biol. Chem.*, 1920–21, xlv, 245.
22. Bloor, W. R., *J. Biol. Chem.*, 1918, xxxvi, 49.
23. De Boer, S., *J. Physiol.*, 1917, li, 211.
24. Loeb, J., *Science*, 1920, lii, 449.
25. Van Slyke, D. D., *Physiol. Rev.*, 1921, i, 141.
26. Campbell, J. M. H., and Poulton, E. P., *J. Physiol.*, 1920–21, liv, 152.
27. Michaelis, L., Die Wasserstoffionenkonzentration, Berlin, 1914, 57.
28. Conway, R. E., and Stephen, F. V., *J. Physiol.*, 1922, lvi, p. xxv.
29. Ryffel, J. H., *Quart. J. Med.*, 1909–10, iii, 221.
30. György, P., and Zunz, E., *J. Biol. Chem.*, 1915, xxi, 511.
31. Kramer, B., Tisdall, F. F., and Howland, J., *Am. J. Dis. Child.*, 1921, xxii, 431.

A STUDY OF CERTAIN PROTEIN PRECIPITANTS.

By ALMA HILLER and DONALD D. VAN SLYKE.

(From the Hospital of The Rockefeller Institute for Medical Research.)

Analytical methods for the separation of proteins from their split-products are based almost entirely on the greater ease with which the proteins, as compared with their products, are thrown out of solution by various coagulants and precipitants. Some of the latter agents do not precipitate all proteins, while some of them are known to precipitate certain proteolytic products; *e.g.*, heat does not coagulate gelatin, alumina cream does not precipitate hemoglobin (1); saturation with ammonium sulfate precipitates not only proteins but also some of the higher albumoses. The various precipitants are used more or less blindly in analyses of complex mixtures such as blood and protein digests.

The present paper reports the results of an attempt to ascertain empirically but somewhat more definitely the manner in which some of these precipitants act towards the proteins and protein derivatives of blood, and of Witte's peptone as a representative mixture of intermediate products. The relative proportions of total nitrogen precipitated, and of the total nitrogen, amino nitrogen, and peptide-bound nitrogen in the filtrates have been studied with seven precipitants. The latter have been applied to blood, peptone solutions, blood plus peptone, and blood plus amino-acids. In order to simplify the amino nitrogen determinations in blood filtrates the blood urea was destroyed with urease before the precipitants were used, and the ammonia formed was removed after precipitation by vacuum distillation of the filtrates.

EXPERIMENTAL.

Preparation of Blood.—1 liter samples of ox blood were collected in large bottles containing 5 gm. each of potassium oxalate. 5 gm. of Squibb's urease were added, and the blood was allowed

to stand 1 hour at room temperature. 1 per cent phenol was added and the blood was kept in the ice box, whence portions were removed as needed.

Preparation of Witte's Peptone Solution.—20 gm. of Witte's peptone were dissolved in water and diluted to a volume of 500 cc. The pH was adjusted to 7.4 and the solution was filtered. In the precipitation experiments this solution was treated as described for blood, except that the preliminary urease treatment was omitted.

Precipitation Methods.—The following methods for precipitating proteins were applied to both ox blood and Witte's peptone:

1. Colloidal Iron and Heat (2).—Before using colloidal iron for precipitating the proteins from whole blood a preliminary test was made to determine the amount of iron required. The procedure was that previously employed by Van Slyke, Vinograd-Villchur, and Losee (2) for plasma, except that we have used sodium sulfate as electrolyte instead of magnesium sulfate. Merck's dialyzed iron (5 per cent Fe_2O_3) and a 20 per cent solution of sodium sulfate were used. The procedure was the following: Into beakers were measured 20 cc. portions of water and 2 cc. portions of ox blood. The contents were heated to boiling and colloidal iron solution was added drop by drop in the amounts designated in Table I. After a few seconds boiling the sodium sulfate solution was added as indicated in the table, and the whole was thrown onto a folded filter. The results are given in Table I.

The proportions used in No. 3 seemed satisfactory for small amounts of blood, but where large volumes were used a slightly cloudy filtrate was obtained. The larger proportions of colloidal iron indicated by No. 2 were apparently required for complete precipitation.

The colloidal iron was used in routine experiments as follows: In a large beaker were mixed 300 cc. of water and 50 cc. of the Witte's peptone solution, or of ox blood which had been treated with urease as described. The mixture was heated to boiling, then 50 cc. of colloidal iron were added drop by drop with stirring. The boiling was continued for a few seconds, while 25 cc. of 20 per cent sodium sulfate were added. The mixture was allowed to cool, then washed into a 500 cc. volumetric flask, and diluted to the mark. It was filtered through a dry folded filter; 250 cc. of the filtrate were measured into a distilling flask, made alkaline to phenolphthalein with sodium hydroxide solution, and concentrated *in vacuo* to about 10 cc.

The residue was neutralized with acetic acid, and diluted to 25 cc. This solution was analyzed in the following manner.

(a) *Total Nitrogen.*—5 cc. portions were analyzed by macro Kjeldahl, using 0.02 N acid and alkali for titration.

(b) *Amino Nitrogen.*—2 cc. portions were analyzed according to Van Slyke (3).

(c) *Peptide Nitrogen.*—To 5 cc. in a hard glass test-tube were added 5 cc. of concentrated hydrochloric acid, the tube was covered with an inverted short, wide tube, and heated for 24 hours at 100° in the steam bath. The contents were then washed into a glass evaporating dish and concentrated almost, but not quite, to dryness. The concentrated filtrates were neutralized to alizarin with 40 per cent sodium hydroxide solution and diluted to 10 cc. 2 cc. were used for amino nitrogen determinations.

TABLE I.

Behavior of Colloidal Iron as Precipitant of Whole Blood.

No.	Ox blood.	Colloidal iron solution (5 per cent Fe_2O_3).	Sodium sulfate, 20 per cent solution.	Remarks.
	cc.	cc.	cc.	
1	2	3	1.5	Filters water-clear and rapidly, but precipitate very bulky.
2	2	2	1.0	Filters water-clear and rapidly, precipitate less bulky.
3	2	1.5	0.75	Filters water-clear and rapidly, precipitate slightly less.
4	2	1.0	0.5	Filtrate yellow and cloudy, precipitate much less.

2. *Tungstic Acid.*—The technique followed was essentially that of Folin and Wu (4).

50 cc. of ox blood or peptone solution were measured into a 500 cc. volumetric flask to which were added 300 cc. of water and 50 cc. of a 10 per cent sodium tungstate solution,[1] and the contents were well mixed. 50 cc. of 2.3 N sulfuric acid were added, the contents were again mixed, were diluted to volume, shaken several times, and after 5 or 10 minutes were filtered through a dry folded filter. Of the filtrate 250 cc. were treated exactly as described under "Colloidal iron."

The final concentration of sodium tungstate is 1 gm. per 100 cc. of final mixture, and it is indicated as "1 per cent tungstate" in Table III.

[1] Primos Chemical Company product.

In the experiments indicated in Table III as "2 per cent tungstate" the conditions were the same, except that twice as much of both tungstate and sulfuric acid were used.

In a separate experiment with Witte's peptone, of which the results are given in Table VI, only one-fifth the above amount of peptone was used, the other details being the same.

3. Trichloroacetic Acid (5).—Ox blood and Witte's peptone were precipitated in 2.5, 5, and 10 per cent trichloroacetic acid.

2.5 Per Cent Trichloroacetic Acid.—50 cc. of blood or peptone solution were diluted with 200 cc. of distilled water, were well mixed, then diluted gradually and with constant shaking, to a volume of 500 cc. with 5 per cent trichloroacetic acid. The mixture was allowed to stand 30 minutes, and was then filtered through a dry folded filter. Of the filtrate 250 cc. were measured into a large beaker and boiled over a free flame for 15 minutes to decompose the bulk of the trichloroacetic acid ($CCl_3COOH = CHCl_3 + CO_2$). The solution was then made alkaline to phenolphthalein with a few drops of sodium hydroxide solution, was concentrated *in vacuo*, and was treated as described under "Colloidal iron."

5 Per Cent Trichloroacetic Acid.—50 cc. of blood or peptone solution were treated as above, except that 10 per cent trichloroacetic acid solution was added instead of 5 per cent. The mixture was allowed to stand 20 minutes. Of the filtrate, 250 cc. were diluted with an equal volume of water, in order to reduce the trichloroacetic acid concentration to 2.5 per cent; since with 5 per cent a slight but measurable hydrolysis of intermediate products may occur when the solution is boiled to decompose the acid. After the dilution the filtrate was boiled and treated like the 2.5 per cent filtrate.

10 Per Cent Trichloroacetic Acid.—50 cc. of blood or peptone solution were treated as above, except that 20 per cent trichloroacetic acid solution was added instead of 5 or 10 per cent. The mixture was allowed to stand 10 minutes. Of the filtrate 250 cc. were diluted 4-fold, and the procedure continued as above.

4. Ethyl Alcohol.—50 cc. of ox blood or peptone solution were diluted to 500 cc. with 95 per cent ethyl alcohol, allowed to stand 24 hours, and then filtered through a dry folded filter. To the filtrate 0.5 cc. of saturated alcoholic solution of zinc chloride was added to precipitate the last traces of protein (6). The solution was well mixed, allowed to stand for a few moments, and again filtered. 250 cc. of the filtrate were made alkaline with sodium hydroxide and concentrated *in vacuo* to a small volume. A little water was added and the solution was again concentrated to drive off the last traces of alcohol, so that the latter would not interfere with the subsequent amino nitrogen determination. The solution was then analyzed as described under "Colloidal iron."

5. Metaphosphoric Acid (7).—The metaphosphoric acid was prepared according to the method of Folin (8) and a 25 per cent solution was made

TABLE II.

Properties of Precipitates and Filtrates Obtained with Different Blood Precipitants.

Precipitant.	Relative volume of precipitate.	Appearance of filtrate.	Rate of filtration.	Volume of filtrate	pH of filtrate.
				cc.	
Colloidal iron.	Very bulky.	Water-clear.	Rapid.	285	6.4
Tungstic acid.	Largest.	" "	Slowest.	340	5.1
Trichloroacetic acid.					
2.5 per cent.	Very bulky.	Water-clear.	Moderately rapid.	395	1.0
5.0 " "	Less bulky.	" "	Very rapid.	435	1.0
10.0 " "	Very small.	" "	Most rapid.	447	1.0
Alcohol.	Very bulky.	Yellow.	Moderate.	370	6.0
Metaphosphoric acid.	Moderately bulky.	Water-clear.	Very slow but faster than tungstic acid.	405	2.1
Picric acid.	Small.	Clear.	Moderately rapid.	130	2.2
Mercuric chloride.	Bulky.	Water-clear.	" "	225	4.4
				(Total 300)	

up just before using. Into a 500 cc. volumetric flask were measured 200 cc. of water, 50 cc. of ox blood, or of peptone solution, and 30 cc. of the 25 per cent solution of metaphosphoric acid. The contents were well mixed and allowed to stand 1 hour, then diluted to volume with water and filtered through a dry folded filter. The remaining procedure was the same as that described under "Collodial iron."

TABLE III.

Precipitations of Witte's Peptone.

Precipitant.	Peptone in 100 cc. of precipitation mixture.	Peptone N in 100 cc. of precipitate mixture.	Percentage of original peptone N in filtrate as				Volume of filtrate from 100 cc. of mixture.	pH of filtrate.
			Total filtrate N.	Amino N.	Peptide N.	Undetermined N.		
	gm.	*gm.*	*per cent*	*per cent*	*per cent*	*per cent*	*cc.*	
None...................	0.400	0.0584	100.0	10.4	62.5	27.1	100	7.4
Trichloroacetic acid.								
2.5 per cent........	0.400	0 0584	85.4	10.5	52.3	22.6	91	> 1
5 " "........	0.080	0 0117		10.5	51.5			
5 " "........	0.400	0.0584	77.9	9.7	46.3	21.9	92	> 1
10 " "........	0 400	0.0584	62.5	9.4	38.1	14.9	94	> 1
HPO₃.....................	0.400	0 0584	67.8	8.3	40.4	19.1	84	1.8
HgCl₂	0 400	0.0584	71.2	8.2	38.9	24.3	83	4.7
Colloidal iron...........	0.400	0.0584	55.2	7.0	29.7	18.5	66	3.6
Picric acid.............	0 400	0.0584		5.3	19.0		80	
Alcohol................	0.400	0.0584	29.4	4.8	21.3	4.6	88	5.7
Tungstate.								
1 per cent...........	0 080	0.0117		5.3	26.7			
2 " "...........	0 080	0 0117		5.3	27.9			
2 " "...........	0 400	0.0584		4 6	17.6			
1 " "...........	0 400	0 0584	26.7	4 0	16.2	6.5	89	2.8

6. *Picric Acid.*—50 cc. of blood were diluted to 500 cc. with saturated aqueous picric acid solution, allowed to stand 25 minutes, and filtered. 250 cc. of the filtrate were treated as described under "Collodial iron," the final dilution being to 50 cc. instead of 25, in order to avoid separation of an inconvenient bulk of picrate crystals.

7. *Mercuric Chloride.*—This precipitant was used essentially according to the technique of Gettler and Baker (9). Into a 500 cc. Erlenmeyer flask were measured 50 cc. of blood, or peptone solution, 50 cc. of water, 100 cc. of 5 per cent hydrochloric acid, and 100 cc. of 5 per cent

mercuric chloride, making a total volume of 300 cc. The solution was well
mixed and filtered. 150 cc. were treated with hydrogen sulfide, and the
mercuric sulfide was filtered and washed. The combined filtrate and wash
water were concentrated *in vacuo* to remove hydrogen sulfide. The con-
tents of the flask were diluted with water, made alkaline to phenolphthalein
with sodium hydroxide solution, and the procedure was continued as
described under "Colloidal iron."

Fig. 1. Nitrogen in the filtrates from three samples of ox blood. The
results from Bloods 1, 2, and 3, respectively, are indicated by three lines in
order from left to right, in the case of each precipitant, except HgCl₂,
which was tested only with Blood 2. For picric acid the total nitrogen
figures are omitted, since the nitrogen content of the precipitant renders
its filtrates unsuited for the Kjeldahl estimation.

The results obtained with three samples of ox blood are shown
by Fig. 1, those with Witte's peptone by Fig. 2 and Table III.
The figures for total nitrogen in blood filtrates were obtained after
the urea had been removed, and therefore represent the non-
protein, non-urea nitrogen.

Amino-Acids Added to Blood.—A solution of mixed monoamino-
acids made from the phosphotungstic acid filtrate from hydrolyzed

casein was added to blood, so that each 100 cc. of ox blood contained an additional 22 mg. of amino nitrogen. The results of this experiment are shown in Table IV.

Precipitation of Witte's Peptone Added to Blood.—To 50 cc. portions of blood which had been treated with urease, 10 cc. portions of 4 per cent Witte's peptone solution, in which the pH had been adjusted to 7.4 were added. The proteins were immediately precipitated by the tungstic acid and 5 per cent trichloroacetic acid methods. The peptone solution was not permitted

Fig. 2. Results with Witte's peptone.

to stand in contact with the urease, as the urease contains a peptolytic enzyme which rapidly splits the peptone, and thereby increases the amino nitrogen. The results of the experiment, shown in Table VI, indicate that the presence of blood does not affect the manner in which these two precipitants act on the intermediate products contained in Witte's peptone.

Hydrolytic Effect of Boiling Trichloroacetic Acid on Unprecipitated Intermediate Protein Products.—The trichloroacetic acid filtrates are boiled to decompose the acid, which when heated splits into $CHCl_3$ and CO_2. As it was uncertain whether decom-

TABLE IV.

Recovery of Amino-Acids Added to Blood.

Precipitant.	Amino nitrogen per 100 cc.				Peptide nitrogen per 100 cc			
	Blood.	Blood + amino-acids.	Recovered.*	Added.	Blood.	Blood + amino-acids.	Recovered.*	Added.
	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.
Trichloroacetic acid, 2.5 per cent......................	9.15 9.82	30.89 30.75			8.92 9.83	8.71 10.39		
Average....................	9.49	30.82	21.18	22.00	9 38	9 55	+0.17	1.0
Trichloroacetic acid, 5 per cent......................	8.48 8.80	32.50 30.05			5.35 6.16	4.20 7.25		
Average....................	8.64	31.28	22.64	22.00	5 76	5.73	−0.03	1.0
Trichloroacetic acid, 10 per cent......................	8.00 8.08	28.95 29.00			5.09 4.63	7.95 8.10		
Average....................	8.04	28.98	20.94	22.00	4.86	8.03	+3.17	1.0
Metaphosphoric acid.........	8.60 8.12	27.83 28.04			4.20 4.85	9.07 9.51		
Average....................	8.36	27.94	19.58	22.00	4.53	9.29	+4.76	1.0
Colloidal iron...............	9.45 9.54 8.68	31.35 31.05 30.65			6.81 10.07 6.68	4.50 2.95 3.25		
Average....................	9.22	31.02	21.80	22.00	7.85	3.56	−4.29	1 0

* The figures in this column are calculated as the difference between the average figures for blood plus amino-acids and for blood alone.

The duplicate figures are from the filtrates of different precipitations and themselves represent averages obtained from aliquot parts of the same filtrate. The differences in the results from each aliquot were relatively negligible (see Table VII). Differences in this table exceeding 0.3 gm. of total or of amino + peptide N, or 0.2 mg. of amino N, are due to failure to obtain exactly identical amounts in duplicate filtrates, not to errors in the final analyses.

TABLE IV—*Concluded*.

Precipitant.	Amino nitrogen per 100 cc.				Peptide nitrogen per 100 cc.			
	Blood.	Blood + amino acids.	Recovered.*	Added.	Blood.	Blood + amino acids.	Recovered.*	Added.
	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.
Picric acid....................	9.23	30.40			4.27	6.34		
	9.29	30.85			5.19	6.75		
Average....................	9.26	30.63	21.37	22.00	4.73	6.55	+1.82	1.0
Alcohol.....................	5.44	21.36			1.37	1.09		
	5.84	21.85				1.58		
Average....................	5.64	21.61	15.97	22.00		1.34	−0.03	1.0
Tungstic acid...............	9.63	31.22			5.03	4.78		
	9.98	31.25			3.52	5.60		
Average....................	9.81	31.24	21.43	22.00	4.28	5.19	+0.91	1.0

position occurs before the acid exerts an appreciable hydrolytic effect on such intermediate products as are in the filtrate, the following experiment was performed in order to test the point. The experiment was made with the filtrate from Witte's peptone rather than from blood, because the intermediate products are much more abundant in the peptone filtrate.

25 cc. portions of a 20 per cent solution of Witte's peptone were precipitated with equal volumes of 5, 10, and 20 per cent trichloroacetic acid. 3 cc. of each filtrate were neutralized with sodium hydroxide and diluted to a volume of 10 cc. 30 cc. of each filtrate were boiled in an open beaker over a free flame for 15 minutes to decompose the trichloroacetic acid, and were then diluted to 100 cc.

The amino nitrogen contents of the solutions were determined. The results as recorded in Table V showed no measurable hydrolysis as the result of boiling the peptone with trichloroacetic acid in a concentration of 2.5 per cent, but did show measurable hydrolysis by 5 and 10 per cent trichloroacetic acid.

Effect of Precipitation Time on the 5 Per Cent Trichloroacetic Acid Method.—In order to discover whether long standing after

TABLE V.

Precipitation of Blood Plus One-Fifth Its Volume of 4 Per Cent Witte's Peptone.

Method.	Amino nitrogen.				Peptide nitrogen.			
	In filtrate from 100 cc. blood.			In filtrate of peptone precipitated in absence of blood.*	In filtrate from 100 cc. blood.			In filtrate of peptone precipitated in absence of blood.*
	Blood.	Blood + 1/5 volume 4 per cent peptone.	Recovered peptone amino N.		Blood.	Blood + 1/5 volume 4 per cent peptone.	Recovered peptide N of peptone.	
	mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
Trichloroacetic acid, 5 per cent..	7.23	20.33			5.42	64.47		
	7.23	20.73			5.30	62.97		
Average..........	7.23	20.53	13.30	12.65	5.36	63.72	58 36	60.10
Tungstate, 1 per cent.............	10.24	16.80			9.69	39.40		
	10.38	16.85			7.54	38.95		
Average..........	10.31	16.82	6.51	6.20	8.61	39.17	30.56	31 10

* These figures are calculated from Table III.

TABLE VI.

Hydrolytic Effect of Boiling Filtrate from Peptone Solution with 2.5, 5, and 10 Per Cent Trichloroacetic Acid.

Concentration of trichloro-acetic acid.	Amino N per gram of peptone.			
	Filtrate not boiled.	Filtrate boiled 15 minutes.	Increase.	
per cent	*mg.*	*mg.*	*mg.*	*per cent*
2.5	13.66	13.60	0.00	0.0
5.0	12.53	13.17	0.64	5.1
10.0	10.16	11.45	1.29	12.7

precipitation with 5 per cent trichloroacetic acid altered the results, the effect was tested both on blood and on Witte's peptone solution, the mixtures being allowed to stand 15 minutes and 24 hours,

respectively, before filtration. The results are shown in Table VII. A very slight transformation of peptide to amino nitrogen may have occurred during the longer period, but the change hardly exceeds the experimental error.

Constancy of Results by the Trichloroacetic Acid Method.—In order to determine the limit of constancy in this method, several precipitations of the same blood were made with 2.5 and 5 per cent trichloroacetic acid. The results are shown in Table VIII.

TABLE VII

Effect of Precipitation Time with 5 Per Cent Trichloroacetic Acid.

Solution.	Precipitation time.	N in filtrate from 100 cc. solution.	
		Amino N.	Amino N + peptide N.
	hrs.	*mg.*	*mg.*
Witte's peptone, 4 per cent........	$\frac{1}{4}$	64.8	335
		64.2	335
" " 4 " "	24	66.4	333
		64.5	335
Ox blood.........................	$\frac{1}{4}$	7.93	12.54
		7.99	12.95
" "	24	8.15	12.20
		8.50	13.18

DISCUSSION OF RESULTS.

Results with Peptone.—From the results with Witte's peptone it appears that tungstic acid and picric acid are distinguished by the relative completeness with which they precipitate protein intermediate products, without precipitating amino-acids. Trichloroacetic acid on the other hand, particularly in solutions more dilute than 5 per cent, permitted nearly all of these products to pass into the filtrate.

It appears, therefore, that trichloroacetic acid is especially fitted for use with solutions of partially digested proteins when it is desired to remove the proteins, and to regain in their filtrates not only the amino-acids, but also a maximum proportion of the intermediate products such as "albumoses" and "peptones." Tungstic and picric acids appear better fitted for experiments in which it is desired to precipitate the intermediate products as completely as possible.

TABLE VIII.

Constancy of Results with Ox Blood by Precipitation with 2.5 and 5 Per Cent Trichloroacetic Acid.

Precipitant.	N in 100 cc. blood.				Deviation from average of 4 filtrates.			
	Total N.*	Amino N.	Amino N. + peptide N.	Peptide N.	Total N.	Amino N.	Amino N. + peptide N.	Peptide N.
	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.
Trichloroacetic acid, 2.5 per cent............	31.14 30.75	9 99 10.09	18.68 18 46					
Average................	30.95	10 04	18 57	8 53	+0.10	+0 26	-0 48	-0.74
Trichloroacetic acid, 2.5 per cent	31.30 31.58	9.81 9.81	18.94 19.16					
Average................	31 44	9.81	19 05	9.24	+0 59	+0 03	±0 00	-0.03
Trichloroacetic acid, 2.5 per cent............	30 47 30.40	9.64 9.56	19.50 19.32					
Average................	30.44	9 60	19 41	9 81	-0.41	-0 18	+0.36	+0.54
Trichloroacetic acid, 2.5 per cent..	30.20 30 90	9.64 9 72	19.16 19 13					
Average................	30.55	9.68	19.16	9.48	-0 30	-0 10	+0 11	+0.21
Average of 4 filtrates	30.85	9.78	19 05	9.27				
Trichloroacetic acid, 5 per cent..............	26.60 26 78	9 67 9.52	15 45 15 32					
Average................	26 69	9 60	15 39	5 79	-0 74	+0 08	+0.48	+0.40
Trichloroacetic acid, 5 per cent	27.59 27 59	9 64 9 63	14 27 14 36					
Average................	27 59	9 64	14 32	4 68	+0 16	+0 12	-0 59	-0 71
Trichloroacetic acid, 5 per cent	27.48 27.30	9 44 9 33	14 59 14 86					
Average................	27 39	9 39	14 73	5 34	-0 04	-0 13	-0 18	-0 05
Trichloroacetic acid, 5 per cent	27.88 28.20	9 51 9.36	15 25 15 14					
Average................	28.04	9 44	15 20	5 76	+0 61	-0 08	+0 29	+0 37
Average of 4 filtrates	27.43	9 52	14 91	5 39				

* The total nitrogen figures represent the total nitrogen of the filtrates from blood from which the urea had previously been removed, and represent, therefore, the non-protein, non-urea nitrogen.

Alcohol behaves toward Witte's peptone like tungstic and picric acids, but for reasons discussed below, is not a desirable precipitant for quantitative work.

Metaphosphoric acid, colloidal iron, and mercuric chloride are intermediate between trichloroacetic acid and tungstic acid in the completeness with which they precipitate the intermediate products of Witte's peptone.

Results with Blood.—The average figures obtained with the different precipitants are given in Table IX.

All the precipitants used appear to remove the blood proteins completely. The completeness of the removal is indicated by the

TABLE IX

Average of Results Obtained with Three Ox Bloods.

Precipitant.	Nitrogen per 100 cc. of blood.		
	Total non-protein, non-urea N.	Amino N.	Peptide N.
	mg.	*mg.*	*mg.*
Tungstic acid..............................	28 1	9.2	4.1
Picric acid	*	8.3	4.6
Metaphosphoric acid.............	28 3	7.9	3.9
2.5 per cent trichloroacetic acid.............	28 8	7.9	7.0
Colloidal iron	29 4	7.8	†
5 per cent trichloroacetic acid...............	26.7	7.5	4.9
10 per cent trichloroacetic acid..............	26 1	7.1	· 4.6
Alcohol......................	18 2	4.9	1.4

* Not determined because of nitrogen content of precipitant.

† Not averaged because of inconsistency of results.

lack of high and irregular figures for the total filtrate nitrogen, and in particular for the peptide nitrogen, such as would have been obtained had even slight proportions of the relatively immense amounts of protein nitrogen present escaped precipitation.

Of the amino nitrogen naturally present in blood, all of the precipitants except alcohol permitted similar though not exactly equal amounts (8 ± 1 mg. per 100 cc.) to pass into the filtrates. In the filtrates from alcohol only about two-thirds as much amino nitrogen was found as in the filtrates from the precipitants used in aqueous solution. Mixed monoamino-acids from hydrolyzed casein added to blood were recovered with approximate complete-

ness in all the filtrates except those from alcohol and metaphosphoric acid (Table IV), from which were recovered 73 and 89 per cent, respectively. Apparently when alcohol is used as a precipitant of the blood, about 30 per cent of the free amino-acids present are adsorbed by the coagulated proteins.[2] Our findings in this respect agree with those of Bock (13).

From the peptide nitrogen data it is evident that, unlike Witte's peptone, the bloods examined contained no appreciable amounts of intermediate products precipitated by picric and tungstic acid, but not by 5 or 10 per cent trichloroacetic or metaphosphoric acid. All five of these precipitants yielded nearly the same peptide nitrogen. The bloods did, however, show 2 to 3 mg. of peptide nitrogen per 100 cc. precipitable by the above reagents, but not by 2.5 per cent trichloroacetic acid.

BIBLIOGRAPHY.

1. Marshall, J., and Welker, W. H., *J. Am. Chem. Soc.*, 1913, xxxv. 820. Welker, W. H., and Williamson, C. S., *J. Biol. Chem.*, 1920, xli, 75.
2. Van Slyke, D. D., Vinograd-Villchur, M., and Losee, J. R., *J. Biol. Chem.*, 1915, xxiii, 381.
3. Van Slyke, D. D., *J. Biol. Chem.*, 1913–14, xvi, 121; 1915, xxiii, 407.
4. Folin, O., and Wu, H., *J. Biol. Chem.*, 1919, xxxviii, 81.
5. Greenwald, I., *J. Biol. Chem.*, 1915, xxi, 61.
6. Folin, O., and Denis, W., *J. Biol. Chem.*, 1912, xi, 527.
7. Folin, O., and Denis, W., *J. Biol. Chem.*, 1916, xxvi, 491.
8. Folin, O., *J. Biol. Chem.*, 1919, xxxix, 259.
9. Gettler, A. O., and Baker, W., *J. Biol. Chem.*, 1916, xxv, 221.
10. Van Slyke, D. D., and Meyer, G. M., *J. Biol. Chem.*, 1912, xii, 399.
11. Folin, O., and Denis, W., *J. Biol. Chem.*, 1912, xi, 87; 1912, xii, 141, 253. Folin, O., and Lyman, H., *J. Biol. Chem.*, 1912, xii, 259. Folin, O., and Denis, W., *J. Biol. Chem.*, 1913, xiv, 29. Folin, O., and Morris, L., *J. Biol. Chem.*, 1913, xiv, 509.
12. György, P., and Zunz, E., *J. Biol. Chem.*, 1915, xxi, 511.
13. Bock, J. C., *J. Biol. Chem.*, 1916–17, xxviii, 357.

[2] Van Slyke and Meyer (10) recovered relatively large amounts of added alanine almost completely from the alcoholic filtrate of dog's blood, but with the smaller concentrations of the mixed amino-acids normally present the proportion adsorbed is too great to permit quantitative recovery. The results obtained by ourselves and other authors indicate, however, that a fairly constant fraction of the total amino-acid nitrogen, *viz.* about two-thirds, is regained in the alcohol filtrate, and that this fraction is sufficiently constant to validate the conclusions drawn from comparative results in physiological experiments such as those of Van Slyke and Meyer (10), Folin (11), and Zunz (12).

THE DETERMINATION OF THE THREE DISSOCIATION CONSTANTS OF CITRIC ACID.

By A. BAIRD HASTINGS and DONALD D. VAN SLYKE.

(*From the Hospital of The Rockefeller Institute for Medical Research.*)

Although the first dissociation constant of citric acid has been found by conductivity to be 8.2×10^{-4} by Walden,[1] and 8.0×10^{-4} by Walker[2] at 25°, and by pH determinations 7.9×10^{-4} by Enklaar[3] at 18°, the values of the second and third dissociation constants have not been available. From the data obtained by the electrometric titration of citric acid, and from formulas developed by Van Slyke in a previous paper,[4] we have calculated the three dissociation constants of the acid.

The electrometric titration of 0.1 M citric acid with 1.0 N NaOH was carried out in a titrating electrode vessel recently described by Hastings.[5] To check these determinations the pH values of citric acid-sodium citrate mixtures, 0.1 M with respect to the citrate radical, were also determined in a Clark cell. These determinations were carried out at $20 \pm 0.1°$. Table I and Fig. 1 show the results.

The calculations were performed in the following way. The equation for the dissociation of a weak acid in the presence of its alkali salt, at pH ranges less than 11, is No. 31 of a previous paper;[4] *viz.*,

$$K'_a = \frac{[H^+] (B + [H^+])}{C - (B + [H^+])},$$

where B is the amount of alkali added to the acid Ha, C is the concentration of the acid, γ the degree of dissociation of the salt

[1] Walden, P., *Z. physik. Chem.*, 1892, x, 568.

[2] Walker, J., *J. Chem. Soc.*, 1892, lxi, 708.

[3] Enklaar, J. E., *Z. physik. Chem.*, 1912, lxxx, 617.

[4] Van Slyke, D. D., *J. Biol. Chem.*, 1922, lii, 525.

[5] Hastings, A. B., *J. Biol. Chem.*, 1921, xlvi, 463.

TABLE I.

Electrometric Titration of Citric Acid with NaOH. Solutions 0.1 M with Respect to Citrate.

NaOH	pH*	$[H^+]$	NaOH	pH	$[H^+]$	NaOH	pH	$[H^+]$
mols per liter			mols per liter			mols per liter		
0.0000	2.06	8.70×10^{-3}	0.1578	4.46	3.47×10^{-5}	0.2821	6.17	6.76×10^{-7}
0.0197	2.51	3.09×10^{-3}	0.1775	4.69	2.04×10^{-5}	0.2841	6.25	5.62×10^{-7}
0.0395	2.88	1.32×10^{-3}	0.1973	4.94	1.15×10^{-5}	0.2861	6.32	4.78×10^{-7}
0.0592	3.14	7.25×10^{-4}	0.2170	5.18	6.61×10^{-6}	0.2880	6.39	4.07×10^{-7}
0.0790	3.42	3.80×10^{-4}	0.2368	5.42	3.80×10^{-6}	0.2900	6.54	2.88×10^{-7}
0.0987	3.67	2.14×10^{-4}	0.2564	5.70	1.995×10^{-6}	0.2920	6.58	2.63×10^{-7}
0.1183	3.98	1.05×10^{-4}	0.2762	6.06	8.71×10^{-7}	0.2939	6.83	1.48×10^{-7}
0.1381	4.20	6.31×10^{-5}	0.2782	6.07	8.51×10^{-7}	0.2959	7.17	6.76×10^{-8}
			0.2802	6.14	7.25×10^{-7}			

* Standard solution used for determining potential of calomel cell = 0.1 N HCl. pH assumed = 1.085, 20°. Gas chain consisted of $Pt - H_2 -$ solution X — saturated $KCl - HgCl_2 - Hg$.

Fig. 1.

270

Ba into B^+ and a', and $K'_a = \dfrac{K_a}{\gamma}$. In solutions where the concentration of Ba is small, γ may be assumed to approach 1.0, and K'_a to approach K_a (this condition will be noted in Table III).

TABLE II.

Calculation of K'_1 from the Formula

$$K'_1 = \frac{[H^+]\ (B + [H^+])}{C - (B + [H^+])}$$

without Correction for B_2 and B_3.

$C = 0.1000$

$[H^+]$	B	$B + [H^+]$	$C - (B + [H^+])$	K_1'
8.7×10^{-3}	0.0000	0.0087	0.0913	8.3×10^{-4}
3.09×10^{-3}	0.0197	0.0228	0.0772	9.1×10^{-4}
1.32×10^{-3}	0.0395	0.0408	0.0592	9.1×10^{-4}
7.25×10^{-4}	0.0592	0.0599	0.0401	10.8×10^{-4}
3.80×10^{-4}	0.0790	0.0794	0.0206	14.6×10^{-4}

TABLE III.

Calculation of K'_1 after the Correction of B_1 for B_2 and B_3 by the Formulas

$$B_2 = \frac{K'_2 C}{[H^+] + K_2} \quad \text{and} \quad B_3 = \frac{K'_3 C}{[H^+] + K_3}$$

$$K'_2 = 4.1 \times 10^{-5}$$
$$K'_3 = 3.2 \times 10^{-6}$$
$$C = 0.1000$$

$[H^+]$	Total B	B_2	B_3	B_1	$B_1 + [H^+]$	$C - (B_1 + [H^+])$	K_1
8.7×10^{-3}	0.0000	0.0000	0.0000	0.0000	0.0087	0.0913	8.3×10^{-4}*
3.09×10^{-3}	0.0197	0.0013	0.0001	0.0183	0.0214	0.0786	8.4×10^{-4}
1.32×10^{-3}	0.0395	0.0030	0.0002	0.0363	0.0376	0.0624	7.9×10^{-4}
7.25×10^{-4}	0.0592	0.0054	0.0004	0.0534	0.0541	0.0459	8.5×10^{-4}
3.80×10^{-4}	0.0790	0.0098	0.0008	0.0684	0.0688	0.0312	8.3×10^{-4}

* The value for this first figure is really K_1 rather than K'_1, since no salt is present.

$$\text{Average } K' = 8.3 \times 10^{-4}$$
$$pK_1 = 3.08$$

The values of K'_1, K'_2, and K'_3 were estimated by successive approximations. (We shall refer to the K'_a of the most strongly dissociated acid group as K'_1, of the middle one as K'_2, and of the least dissociated as K'_3.) From the data towards the most acid

and least acid ends of the titration curve (Fig. 1), first approximations of the values of K'_1 and K'_3, respectively, were made (Tables II and IV). From the approximate values of K'_1 and K'_3 thus obtained the amounts of base, B_1 and B_3, bound by the two end-carboxyls over the middle part of the curve, were calculated by Equation 10 of the previous paper;[4] $viz.$, $B = \dfrac{K'\,C}{[H^+]+K'}$. The B_1 and B_3 values thus calculated were subtracted from the total amount of NaOH added (B) to give B_2. $B_2 = B - B_1 - B_3$. From the values of B_2 thus obtained K'_2 was calculated.

TABLE IV.

Calculation of K'_3 from the Formula

$$K'_3 = \frac{[H^+]\,[B]}{C - B}$$

without Correction for B_1 and B_2.

$C = 0.1000$

$[H^+]$	B	$C - B$	K'_3
2.63×10^{-7}	0.0920	0.0080	3.0×10^{-6}
2.88×10^{-7}	0.0900	0.0100	2.6×10^{-6}
4.07×10^{-7}	0.0880	0.0120	3.0×10^{-6}
4.78×10^{-7}	0.0861	0.0139	3.0×10^{-6}
5.62×10^{-7}	0.0841	0.0159	3.0×10^{-6}
6.76×10^{-7}	0.0821	0.0179	3.1×10^{-6}
7.25×10^{-7}	0.0802	0.0198	2.9×10^{-6}
8.51×10^{-7}	0.0782	0.0218	3.0×10^{-6}
8.71×10^{-7}	0.0762	0.0238	2.8×10^{-6}
1.995×10^{-6}	0.0564	0.0436	2.6×10^{-6}
3.80×10^{-6}	0.0368	0.0632	2.2×10^{-6}

From this value of K'_2 and the above mentioned first approximations of K'_2 and K'_3, B_2 and B_3 were estimated, in order to calculate the exact B_1, at the acid end of the curve by the equation $B_1 = B - B_2 - B_3$. From the B_1 values thus obtained, a series of consistent values for K'_1 was obtained (Table III).

The B_3 values at the alkaline end of the curve were then estimated as $B_3 = B - B_1 - B_2$, the B_1 and B_2 values being obtained from the K'_1 and K'_2 values, found as above described. The B_3 values thus obtained yielded a series of consistent values for K'_3 (Table V).

TABLE V.

Calculation of K_3' after the Correction of B_3 for B_1 and B_2 by the Formulas

$$B_1 = \frac{K_1'C}{[H^+] + K_1'} \text{ and } B_2 = \frac{K_2'C}{[H^+] + K_2'}$$

$$K_1' = 8.3 \times 10^{-4}$$
$$K_2' = 4.1 \times 10^{-5}$$
$$C = 0.1000$$

$[H^+]$	Total B	B_1	B_2	B_3	$C - B$	K_3'
2.63×10^{-7}	0.2920	0.1000	0.0994	0.0926	0.0074	3.3×10^{-6}
2.88×10^{-7}	0.2900	0.1000	0.0993	0.0907	0.0093	2.8×10^{-6}
4.07×10^{-7}	0.2880	0.1000	0.0991	0.0889	0.0111	3.3×10^{-6}
4.78×10^{-7}	0.2861	0.1000	0.0990	0.0871	0.0129	3.2×10^{-6}
5.62×10^{-7}	0.2841	0.1000	0.0988	0.0853	0.0147	3.3×10^{-6}
6.76×10^{-7}	0.2821	0.1000	0.0985	0.0836	0.0164	3.4×10^{-6}
7.25×10^{-7}	0.2802	0.1000	0.0983	0.0819	0.0181	3.3×10^{-6}
8.51×10^{-7}	0.2782	0.1000	0.0982	0.0800	0.0200	3.4×10^{-6}
8.71×10^{-7}	0.2762	0.1000	0.0981	0.0781	0.0219	3.1×10^{-6}
1.995×10^{-6}	0.2564	0.0998	0.0954	0.0612	0.0388	3.1×10^{-6}
3.80×10^{-6}	0.2368	0.0996	0.0917	0.0455	0.0545	3.2×10^{-6}

Average $K_3' = 3.2 \times 10^{-6}$
$pK_3' = 5.49$

TABLE VI.

Calculation of K_2' from the Formula

$$K_2' = \frac{[H^+] \ [B_2]}{C - B_2}$$

where B_2 is Calculated from

$$B_2 = B - [B_1 + B_3]$$
$$K_1' = 8.3 \times 10^{-4}$$
$$K_3' = 3.2 \times 10^{-6}$$
$$C = 0.1000$$

$[H^+]$	Total B	B_1	B_3	B_2	$C - B_2$	K_2'
3.80×10^{-4}	0.0790	0.0686	0.0008	0.0096	0.0904	4.0×10^{-5}
2.14×10^{-4}	0.0987	0.0798	0.0015	0.0174	0.0826	4.5×10^{-5}
1.05×10^{-4}	0.1183	0.0888	0.0030	0.0265	0.0735	3.8×10^{-5}
6.31×10^{-5}	0.1381	0.0930	0.0048	0.0403	0.0597	4.3×10^{-5}
3.47×10^{-5}	0.1578	0.0961	0.0084	0.0533	0.0467	4.1×10^{-5}
2.04×10^{-5}	0.1775	0.0977	0.0135	0.0663	0.0337	4.0×10^{-5}

Average $K_2' = 4.1 \times 10^{-5}$
$pK_2' = 4.39$

K'_2 was finally reestimated by using the accurate K'_1 and K'_3 values to calculate the B_1 and B_3 figures of the equation $B_2 = B - B_1 - B_3$. The B_2 values thus obtained yielded a series of consistent values for K'_2 (Table VI).

TABLE VII.

Calculation of K'_2 from the Buffer Value β_2.

$pK'_1 = 3.08$

$pK'_3 = 5.49$

pH	ΔpH	Mean pH	ΔB	$\dfrac{\Delta B}{\Delta pH}$	pH−pK'_1	pH−pK'_3	β_1	β_3	β_2	pH−pK'_2	pK'_2
3.69 3.95	0.26	3.82	0.02	0.0770	0.74	−1.67	0.0301	0.0046	0.0423	−0.49	4.31
3.82 4.08	0.26	3.95	0.02	0.0770	0.87	−1.54	0.0241	0.0063	0.0466	−0.40	4.35
3.95 4.22	0.27	4.09	0.02	0.0741	1.01	−1.40	0.0190	0.0083	0.0468	−0.39	4.48
4.08 4.35	0.27	4.22	0.02	0.0741	1.14	−1.27	0.0116	0.0112	0.0483	−0.37	4.59
4.22 4.48	0.26	4.35	0.02	0.0770	1.27	−1.14	0.0112	0.0147	0.0511	−0.30	4.65
4.35 4.59	0.24	4.47	0.02	0.0834	1.39	−1.02	0.0086	0.0184	0.0564	+0.10	4.37
4.48 4.72	0.24	4.60	0.02	0.0834	1.52	−0.89	0.0063	0.0233	0.0538	+0.23	4.37
4.59 4.84	0.25	4.72	0.02	0.0800	1.64	−0.77	0.0052	0.0288	0.0460	+0.42	4.30
4.72 4.97	0.25	4.85	0.02	0.0800	1.77	−0.64	0.0037	0.0345	0.0415	+0.51	4.34

K'_2 was also calculated from the buffer value $\dfrac{dB}{dpH}$. The total buffer value of the solution at any pH may be expressed as $\beta = \beta_1 + \beta_2 + \beta_3$. β may be evaluated from the titration curve

by calculating $\dfrac{\Delta B}{\Delta pH}$. β_1 and β_3 may be calculated[6] from K'_1 and K'_3 by Equation 35; *viz.*,

$$\beta = 2.3 \left(\frac{K' \, C \, [H^+]}{(K' + [H^+])^2} + [H^+] + [OH'] \right),$$

the [OH'] being negligible in the present case. They may also be estimated graphically by means of Fig. 9 of the previous paper.[4]

Fig. 2.

β_2 is then obtained by difference, and K'_2 calculated either from Equation 22,

$$K'_2 = [H^+] \frac{C - 0.8686 \, \beta_M \pm \sqrt{(0.8686 \, \beta_M - C)^2 - 0.756 \, \beta_M{}^2}}{0.8686 \, \beta_M}$$

or graphically, as described in the previous paper, from Fig. 9. That value of K'_2 is the correct one which is identical with the $[H^+]$ when $\beta_M = 0.575$.

[6] The equation numbers used in the present paper refer to the numbered equations in a former paper.[4]

Values of pK_2' obtained by this method are given in Table VII. The value at that point where β_2 most nearly approaches its maximum of 0.0564, is $pK' = 4.37$.   Other values range from 4.30 to 4.65.   The agreement with the first method of calculation is, we believe, sufficiently good.

The value of the buffer effect of 0.1 M citrate, estimated from the above three pK' values by graphic summation (as in[4] Fig. 8) are given in Fig. 2.

SUMMARY.

The methods for the calculation of the dissociation constants of weak polybasic acids recently outlined[4] have been applied to citric acid.   The values of the three constants have been found to be $K_1' = 8.3 \times 10^{-4}$, $K_2' = 4.1 \times 10^{-5}$, and $K_3' = 3.2 \times 10^{-6}$. The corresponding pK' values are 3.08, 4.39, and 5.49, respectively. The value of K_1' agrees approximately with that of K_1 found by other authors. [1, 2, 3]   The values of K_2' and K_3', because of the overlapping effects of the carboxyl groups, have not been accessible by previous methods of calculation.

THE EFFECT OF ETHER ANESTHESIA ON THE ACID-BASE BALANCE OF THE BLOOD.

By DONALD D. VAN SLYKE, J. HAROLD AUSTIN, and GLENN E. CULLEN.

(From the Hospital of The Rockefeller Institute for Medical Research, New York, and the Department of Experimental Medicine of the University of Pennsylvania, Philadelphia.)

There has been disagreement in the literature concerning the effect of ether anesthesia on the acid-base balance. Menten and Crile (1915) reported a fall in the blood pH in rabbits. Caldwell and Cleveland (1917) found a consistent fall in the plasma bicarbonate as the result of ether anesthesia in patients. Carter (1920) found a similar fall in etherized dogs. If observations by Caldwell and Cleveland and Carter on blood bicarbonate could be linked with Menten and Crile's pH determinations on rabbits, the combined data, indicating lowered [$BHCO_3$] and lowered pH, respectively, could indicate nothing but an uncompensated alkali deficit.

However, Y. Henderson and Haggard (1918) in experiments on dogs showed that the blood alkali reserve, as measured by the CO_2 capacity, could be lowered by experimental hyperventilation. The latter lowers the free H_2CO_3 of the blood, with the result that as shown by Milroy (1914), an increased pH results. The results of Henderson and Haggard indicated that as a secondary effect there may also be a shift of alkali from blood to tissues, or of acid in the reverse direction. Such an effect could be explained as an attempt to lessen the increased pH resulting from abnormal lowering of H_2CO_3. The result of such a condition is indeed a lowered bicarbonate, but, it is primarily due to H_2CO_3 deficit, or CO_2 alkalosis (exemplified by Areas 2 and 3 of Fig. 1, Van Slyke, 1921, b), rather than to alkali deficit resulting from entrance of non-volatile acids (exemplified by Areas 6 and 9).

277

Hasselbalch and Lundsgaard (1912) had showed that merely tying rabbits down may result in a gradual fall in blood pH; so there appeared to be some reason for being uncertain that Menten and Crile's observed fall in blood pH was due to etherization. Henderson and Haggard reported no pH determinations, but their results with ether seemed most readily interpreted by the CO_2 deficit explanation.

It appeared that a decision between the two diametrically opposed interpretations of the observed fall in blood $[BHCO_3]$ could be reached only by observations in which both $[BHCO_3]$ and pH were determined. In a preliminary report (1920) the present authors showed that the pH falls (in agreement with Crile and Menten) whenever it undergoes any change during ether anesthesia. Fall in bicarbonate was consistently observed.

Since the appearance of the above report Collip (1920), experimenting with etherized dogs, calculated the blood pH from the CO_2 tension of the alveolar air and the CO_2 content of the blood. He found this method not entirely satisfactory, but indicative of a fall in blood pH. Recently Atkinson and Ets (1922) have again determined both CO_2 content and pH (the latter by the Dale and Evans colorimetric dialysis method) in the blood of etherized dogs, and have found a fall in both, with recovery after discontinuance of anesthesia.

In the present paper we report the results of six representative experiments. The pH changes in some cases were determined electrometrically, in others colorimetrically, and in others by calculation from the $[BHCO_3] : [H_2CO_3]$ ratio obtained by equilibration with known CO_2 tensions. The bicarbonate was determined both gasometrically and by titration.

The depth and time of anesthesia have been varied. The results concerning the acid-base change have been consistent, and we have consequently felt justified in reporting in detail only a sufficient number of experiments to indicate fairly the nature of the data obtained.

Methods.

Animals.--Large dogs (10 to 15 kilos) were used so that large samples of blood (25 to 50 cc.) might be taken. The dogs were fed as usual the day preceding the experiment but received no food on the day of the experiment.

Bleeding.—The blood was drawn from the left ventricle (unless stated otherwise) through a 4 inch, 16 gauge lumbar puncture needle into a tube under oil. When oxalated blood was desired the tube was previously coated with neutral potassium oxalate to make 0.3 per cent. If defibrinated blood was wanted, the blood was defibrinated under oil by gentle stirring. Since it is usually impossible to avoid some hemolysis in oxalated plasma of dog's blood, it was decided in the later experiments to utilize the true serum of the blood as drawn, allowing coagulation to occur spontaneously while centrifuging.

In centrifuging for true plasma or true serum the blood was drawn directly into a centrifuge tube of the proper size, containing mineral oil. The glass delivery tube was withdrawn as the blood ran in, so that the tube was completely filled with the blood except for a layer of paraffin oil about 1 cm. deep. A 1-hole rubber stopper was inserted, with complete expulsion of the oil. The hole was closed with a glass plug and the tube centrifuged at once. After centrifuging, the glass plug was removed, and from a pipette oil was allowed to flow through the hole in the stopper as the stopper was removed. It is shown elsewhere that such precautions are necessary to prevent loss of CO_2 during centrifugation. The plasma or serum was then transferred without loss of CO_2 to Haldane sampling tubes over mercury or to tubes under oil.

Anesthesia.—The animal was anesthetized in all cases by the drop method with a few layers of gauze. This method was continued throughout the anesthesia unless otherwise stated.

Ventilation Rate.—Ventilation rate was measured in the control periods and during the early part of anesthesia by the use of a closely fitting, well greased rubber mask that fitted and enclosed the entire muzzle of the dog. A Y-tube close to the mask led to two 1 inch aluminum Siebe Gorman valves. The expired air was collected and measured in a 65 liter spirometer.

After the animal was anesthetized a cannula was tied in the trachea and connected to the Y-tube and valves. The intake of air was through a vessel containing gauze onto which ether could be dropped to maintain the anesthesia.

Equilibration of Blood or Serum with CO_2.—The blood was introduced into a partially evacuated tonometer which contained

the required amount of CO_2 and had an oxygen tension approximately atmospheric at $38°$. The tonometer was then rotated in a water bath at $38°$ until equilibration was complete. The equilibration was either repeated or the CO_2 tensions corrected for the CO_2 taken up or given off by the blood. The blood was then transferred, with precautions to prevent loss of CO_2, to Haldane sampling tubes over mercury, or, under oil, to centrifuge tubes. The centrifuging was carried out with the precautions described above.

Hydrogen Ion Concentration Measurements.—Electrometric.— These determinations were made at $20°$ on whole blood with the Clark cell using Hasselbalch's refilling technique. Although the determinations were made with the cells present, Parsons (1919–20) has shown that the pH determined is that of the plasma.

*Colorimetric.—*The colorimetric pH measurements were made at room temperature with phenol red in the diluted plasma or serum by the method recently described by Cullen (1922). The correction used was -0.34, to reduce pH colorimetrically determined at $20°$ to that electrometrically found at $38°$. This correction may subsequently be altered, but such alteration would not affect the pH changes observed.

*Analytical Methods.—*The carbon dioxide determinations were made usually in duplicate on 1 cc. samples by Van Slyke's method with either the fine bore constant pressure apparatus (Van Slyke and Stadie, 1921), or the constant volume apparatus (Van Slyke, 1921, *a*). In Experiment 5, in addition to the gasometric determination of the total CO_2 content, the $BHCO_3$ was determined directly by the titration method (Van Slyke, 1922, *b*).

Calculation.

In discussion of the calculations we shall use the following abbreviations: mM. for millimolar; $[CO_2]$, $[BHCO_3]$, $[H_2CO_3]$ for mM. concentration of CO_2, $BHCO_3$, and H_2CO_3, respectively; p_{CO_2} for CO_2 tension in millimeters of mercury.

In these experiments we have obtained by analysis two or more of the following data: $[CO_2]$ of the blood, plasma, or serum, as drawn from the left ventricle or femoral artery; $[CO_2]$ of the oxygenated blood, true plasma, or serum after equilibration of the blood at $38°$ in tonometers at known p_{CO_2}; the pH

determined electrometrically or colorimetrically on the plasma or serum as drawn, or upon the true plasma or serum after equilibration at known p_{CO_2}. Our problem has been to determine from these data the changes that have occurred, *in vivo*, in the alkaline reserve, CO_2 tension, and pH of the blood.

In Experiments 1 to 3 we have data on the $[CO_2]$ of blood, or the plasma of blood, equilibrated at various CO_2 tensions, and the $[CO_2]$ of the arterial blood, plasma, or serum, as drawn. From the $[CO_2]$ and p_{CO_2} values determined in the equilibrated blood or its true serum, we have estimated the pH values of the blood plasma, and plotted them as abscissæ against the $[CO_2]$ values as ordinates. The resulting curves are almost exactly straight lines.[1] By interpolating the $[CO_2]$ values observed in the blood as drawn on these lines we have determined the pH of the blood as drawn. From the pH thus interpolated and the $[CO_2]$, the p_{CO_2} and $[BHCO_3]$ were calculated.

In constructing our $[CO_2]$, pH curves we have in each case drawn the mean straight line through the points determined on the equilibrated bloods. It appears that such a line compensates for errors in individual determinations and is a more accurate representation of the correct $[CO_2]$, pH curves than a broken line drawn through the individual points. The pH values corrected by means of the straight line graphs thus drawn are indicated in the tables as "Rectified pH" points. It will be noted that the

[1] The fact that when blood pH is plotted against $[CO_2]$ straight line curves are obtained over the physiological range of CO_2 tensions was first noted by Lewis, Cotton, Barcroft, Milroy, Duften, and Parsons (1916). McLean, Murray, and Henderson (1920) found straight line curves also when they plotted pH values against $[BHCO_3]$. The approximately linear character of these curves is attributable to the, as one might say, accidental fact, that the COOH groups of hemoglobin are so arranged that the buffer value (Van Slyke, 1922, *a*) of blood over the physiological pH range is practically constant.

Both curves cannot be exactly linear, for the $[H_2CO_3]$ area which separates $[CO_2]$ from $[BHCO_3]$ is curved. However, this area above pH 7 is relatively narrow, so that it does not prevent both the $[CO_2]$ and $[BHCO_3]$ curves from approximating the linear form. Which of the two does so most closely is at present uncertain, since both appear, over the range pH 7 to 7.8 to vary from straight lines by no more than the experimental errors heretofore connected with pH and $[CO_2]$ measurements.

rectifications fall within the limit of 0.02 pH, which may be taken as the limit of experimental error.

The calculations of pH from $[CO_2]$ and p_{CO_2} values obtained from equilibration data, and of p_{CO_2} and $[BHCO_3]$ from the determined $[CO_2]$ in the blood as drawn and the pH found by graphic interpolation, were performed by means of Hasselbalch's (1917) equation, $pH = pK' + \log \dfrac{[BHCO_3]}{[H_2CO_3]}$. For the above respective calculations the equation was rearranged into the following forms by steps given in another place (Austin, Cullen, Hastings, McLean, Peters, and Van Slyke, 1922).

For whole blood, $pH = 6.20 + \log \dfrac{[CO_2] - 0.0300\ p_{CO_2}}{0.0300\ p_{CO_2}}$,

$$p_{CO_2} = \frac{1}{0.0300} \times \frac{[CO_2]}{10^{pH - 6.20} + 1}, \quad [BHCO_3] = [CO_2] \times \frac{10^{pH - 6.20}}{10^{pH - 6.20} + 1}.$$

For plasma, $pH = 6.10 + \log \dfrac{[CO_2] - 0.0318\ p_{CO_2}}{0.0308\ p_{CO_2}}$,

$$p_{CO_2} = \frac{1}{0.0318} \times \frac{[CO_2]}{10^{pH - 6.10} + 1}, \quad [BHCO_3] = [CO_2] \times \frac{10^{pH - 6.10}}{10^{pH - 6.10} + 1}.$$

The values of pK', 6.10 for plasma and 6.20 for whole blood, are the averages determined on a number of dog bloods. They may later be corrected in the second decimal places but such correction would not significantly affect the *changes* in pH, p_{CO_2}, and $BHCO_3$ calculated in our experiments.

In terms of millimolar concentration, $[H_2CO_3] = \dfrac{\alpha CO_2 \quad p_{CO_2}}{0.0224 \times 760}$. This value is $0.0300\ p_{CO_2}$ when α_{CO_2} (the solubility coefficient of CO_2) is 0.511, as in whole blood (Bohr, 1905), while it is $0.0318\ p_{CO_2}$ when α_{CO_2} is 0.541, as in serum.

Changes in alkali reserve have been measured as changes in the bicarbonate content of the blood estimated at a given pH; *viz.*, the pH observed before ether was administered. This pH was interpolated on the pH, $[CO_2]$ graph obtained after ether, and the $[BHCO_3]$ was calculated for the point thus located. At constant pH, the change in blood $[BHCO_3]$ expresses directly the change in the excess of total base over acids other than H_2CO_3 (Van Slyke, 1921, *b*, p. 169), and because of this advantage in definiteness of interpretation, we have chosen to measure bicarbonate changes at a given pH rather than at a given p_{CO_2}.

The mode of calculation above outlined, and used in Experiments 1, 2, and 3, is illustrated by Fig. 1, by data from Experiment 3.

In Experiments 4, 5, and 6 the data directly determined have been the pH and the $[CO_2]$ of the blood as drawn. From these the $[BHCO_3]$ and p_{CO_2} have been calculated by the equations previously given.

Fig. 1.

Extrapolation of the $[BHCO_3]$ value of the bloods drawn after etherization to the values which those bloods would have under CO_2 tension such as would restore pH to its initial value, was necessary in order to measure the alkali reserve change at constant pH. For constructing the pH, $[BHCO_3]$ lines necessary for the extrapolation in these experiments, there were available no graphs experimentally determined from equilibration data, since data on each blood were determined only at one point, with CO_2 tension as it existed when the blood was drawn. We have ac-

Fig. 2.

cordingly constructed two graphs in each case, using the two extreme limits of the $\dfrac{d[BHCO_3]}{dpH}$ slope as determined in a number of dog bloods from experiments of our own and from experiments in the literature. These limits are $\dfrac{d[BHCO_3]}{dpH} = -20$ to -28, [BHCO₃] being expressed in millimol units. The two sets of alkali reserve changes estimated by extrapolation with these two slopes are indicated, for Experiments 4, 5, and 6, in Fig. 2 by two lines, with a shaded area between them. The width of this area indicates the maximum error introduced by this method of extrapolation.

In Fig. 2 we have plotted the results as changes in alkaline reserve, CO_2 tension, pH, and ventilation rate from the initial values determined before anesthesia.

In our tables we have given the absolute values of our data.

Experiments and Results.

The details of the six experiments are shown in the protocols and tables and the results are given graphically in Fig. 2. In Experiments 1 and 2 the anesthesia was as deep as possible. In Experiments 3 and 4 it was as light as possible, the animal making spontaneous movements from time to time. In Experiments 5 and 6 the animals were given an uneven anesthesia, being at times deeply under and at times almost out of ether but with the anesthesia becoming more evenly settled at about the middle of the second stage as the experiment progressed.

In Experiments 1 to 4 no special precaution was taken to prevent fall in body temperature and as indicated in the protocol of Experiment 4 some cooling occurred. In Experiments 5 and 6 fall in temperature was prevented by covering the animal with a blanket throughout the experiment. The results, however, were substantially the same.

Ventilation rate was studied only in the last two experiments. There is continual fluctuation in the ventilation rate but in both of these experiments there is a tendency to its progressive increase in spite of the fact that in the latter part of the experiment the animals were held in the middle of the second stage of anesthesia.

SUMMARY.

In all the experiments it will be seen that the pH of the blood fell either at once or in a short time after the anesthesia was begun, and in the last three experiments it will be seen that although the pH remains low or continues to fall, the alkaline reserve also continues to fall. In no case was any rise of pH above the initial value observed. In all but one of the experiments the CO_2 tension was increased.

The evidence indicates that these changes do not occur as a compensatory mechanism to balance an acapnia. A true acidosis occurs with increase of the hydrogen ion concentration of the blood and fall of the alkaline reserve, due either to introduction of acid into the blood or to withdrawal of base from it.

Experiment 1.

Young male. 10.12 a.m. first bleeding, left ventricle; 2.15 p.m. ether started, drop method, deep anesthesia; 3.15 p.m. second bleeding, left ventricle.

Blood Equilibrated at 38° (Defibrinated).

Sample.	Time drawn.	CO_2 tension.	Total [CO_2].	pH	
				Calculated.	Rectified by graph.
		mm.	*mM.*		
1	Before ether.	18.7	10.9	7.465	7.465
		37.4	15.2	7.300	7.315
		56.2	18.8	7.206	7.193
		74.9	20.9	7.118	7.118
2	After 60 minutes deep etheriza-tion.	56.2	14.2	7.069	7.063*
		74.8	16.0	6.990	7.002*

* Graph drawn parallel to that of blood before ether.

Blood as Drawn.

Sample.	Time drawn.	Determined.			Calculated.				
		Total [CO₂].	O₂ content.	O₂ capacity.	Total [CO₂] oxygenated at same pH.*	pH by interpolation of CO₂.	CO₂ tension	[BHCO₃]	
								As drawn.	At initial pH.
		mM.	mM.	mM.	mM.		mm.	mM.	mM.
1	Before ether.	16.4	9.70	10.15	16.2	7.27	42	14.9	14.9
2	After ether.	15.9	8.55	11.17	14.5	7.05	60	12.7	9.2†

* $\dfrac{d\ CO_2}{d\ O_2} = 0.52$ at constant pH (unpublished data).

† From extrapolated CO_2 (see Fig. 1).

Experiment 2.

Young male (same as Experiment 1). 9.25 a.m. first bleeding, left ventricle; 9.49 a.m. ether started, drop method, deep anesthesia; 10.51 a.m. second bleeding, left ventricle.

True Plasma from Oxalated Blood Equilibrated at 38°.

Sample.	Time drawn.	CO₂ tension.	Total [CO₂].	pH	
				Calculated.	Rectified by graph
		mm.	mM.		
1	Before ether.	40	18.2	7.224	7.224
		60	21.5	7.112	7.112
2	After 62 minutes deep ether.	20	9.8	7.257	7.257
		70	18.5	6.960	6.960

True Plasma (Oxalated) as Drawn.

Sample.	Time drawn.	Total [CO₂].	Calculated.			
			pH by interpolation of [CO₂].	CO₂ tension.	[BHCO₃]	
					As drawn.	At initial pH.
		mM.		mm.	mM.	mM
1	Before ether.	18.2	7.22	40	16.9	16.9
2	After 62 minutes deep ether.	17.2	7.00	59	15.3	10.0*

* From interpolated [CO₂].

Experiment 3.

Young male (same as Experiments 1 and 2). 10.45 a.m. first bleeding, left ventricle; 11.55 a.m. ether started, lightest possible anesthesia, drop method; 12.25 to 12.37 p.m. breathing very violently; 12.37 to 1.00 p.m. anesthesia very light, regular deep breathing, 33 per minute; 1.00 p.m. second bleeding, left ventricle.

Oxalated Blood Equilibrated at 38°.

Sample.	Time drawn.	CO_2 tension.	Total [CO_2].	pH	
				Calculated.	Rectified by graph.
		mm.	*mM.*		
1	Before ether.	21.8	15.1	7.545	7.550
		50.8	21.0	7.305	7.230
		72.5	23.4	7.190	7.195
2	After 65 minutes light ether.	21.4	10.0	7.367	7.360
		50.8	15.6	7.165	7.180
		72.5	18.9	7.086	7.070

Blood as Drawn (Oxalated).

Sample.	Time drawn.	Total [CO_2].	pH by interpolation	Calculated.		
				CO_2 tension.	[$BHCO_3$]	
					As drawn.	At initial pH.
		mM.		*mm.*	*mM.*	*mM.*
1	Before ether.	18.6	7.40	41	17.7	17.7
2	After 65 minutes light ether.	18.9	7.07	75	17.1	8.3*

* From [CO_2] interpolated at pH 7.40.

Experiment 4.

Young male. 1.10 p.m. first bleeding, left ventricle; 1.2S p.m. ether started, drop method, light anesthesia; 1.55 p.m. second bleeding, right femoral artery; 2.1S p.m. third bleeding, right femoral artery; 2.42 p.m. fourth bleeding, left femoral artery. Rectal temperature: initial and maximum 39.9; final and minimum 37.3.

True Serum as Drawn (Spontaneous Coagulation).

Sample.	Time drawn.	Determined.			Calculated.			
		[BHCO₃] titration.	Total [CO₂] gasometric.	Colorimetric pH.	CO₂ tension.	[BHCO₃] as drawn from gasometric CO₂.	[BHCO₃] at initial pH.	
							$\dfrac{d[\text{BHCO}_3]}{d\,\text{pH}} = -26$	$\dfrac{d[\text{BHCO}_3]}{\frac{dB}{d\,\text{pH}}\text{ observed}} = -28$
		mM.	*mM.*		*mm.*	*mM.*	*mM.*	*mM.*
1	Before ether.	21.5	23.0	7.29	54	21.7	21.6	21.6
2	After 27 minutes light ether.	12.S	16.S	7.29	40	15.9	16.0	16.0
3	After 50 minutes light ether.	12.S	13.S	7.23	37	12.9	11.7	11.2
4	After 74 minutes light ether.	12.6	13.5	7.07	51	12.3	6.3	6.3

Experiment 5.

Young male. 10.02 a.m. first bleeding, left ventricle; 10.10 a.m. ether started, drop method; 10.27 a.m. cannula in trachea, anesthesia kept in middle of second stage; 11.00 a.m. second bleeding, left femoral artery; 11.47 a.m. third bleeding, right femoral artery. Rectal temperature: initial 38.2°; final 38.3°; maximum 38.6°; minimum 38.2°C.

Sample	Time drawn.	Total [CO₂] oxalated blood as drawn.	Colorimetric pH. True serum as drawn.	CO₂ tension.	[BHCO₃] as drawn.	[BHCO₃] at initial pH. $\frac{d[BHCO_3]}{d\,pH} = -20$	$\frac{d[BHCO_3]}{d\,pH} = -28$
		mM.		*mm.*		*mM.*	*mM.*
1	Before ether.	21.70	7.44	39	20.5	20.5	20.5
2	After 50 minutes varied ether.	16.78	7.20	47	15.4	10.6	8.7
3	After 97 minutes varied ether.	14.03	7.18	45	12.7	7.3	5.2

Time.	Ventilation rate.	Per cent Δ.
	liters per min.	
Day before.	5.6	
9.48 a.m.	3.9	
Mean before ether.	4.8	±0
10.35 a.m.	11.0	+129
10.52 "	10.4	+118
11.07 "	4.1	− 15
11.17 "	6.5	+ 35
11.20 "	13.6	+183
11.43 "	9.9	+106
12.14 p.m.	10.0	+108

Experiment 6.

About 4 year old male. 10.50 a.m. first bleeding, left ventricle; 10.56 a.m. ether started, drop method, kept about mid-second stage; 11.15 a.m. second bleeding, left femoral artery; 11.32 a.m. tracheal cannula introduced; 12.13 p.m. third bleeding, right femoral artery. Rectal temperature: initial $38.2°$; final $38.3°$; maximum $38.5°$; minimum $38.2°C$.

Separate Serum Equilibrated at 38°.

Sam- ple	Time drawn.	Atmospheric CO_2 tension.	Serum.			Calculated from colorimetric pH			pK'
			Total [CO_2].	Electrometric pH 38°.	Colorimetric pH.	[H_2CO_3]	[$BHCO_3$]	$\log \frac{[BHCO_3]}{[H_2CO_3]}$	
		mm	$mM.$			mM	$mM.$		
1	Before ether.	40.4	25.52	7.38	7.39	1.28	24.24	1.277	6.11
3	After 77 minutes varied ether.	39.4	18.65	7.19	7.19	1.25	17.40	1.144	$\dfrac{6.05}{\text{Mean } 6.08}$

True Serum as Drawn.

Sample.	Time drawn.	Total [CO_2.]	Colori- metric pH.	Calculated (using pK' = 6 08).		[BHCO$_3$] at initial pH.	
				CO_2 tension.	[BHCO$_3$] as drawn.	$\dfrac{d [BHCO_3]}{d \text{ pH}} = 20$	$\dfrac{d [BHCO_3]}{d \text{ pH}} = 28$
		$mM.$		$mm.$	$mM.$	$mM.$	$mM.$
1	Before ether.	25.32	7.36	40	24.06	24.1	24.1
2	After 19 minutes ether.	22.20	7.20	49	24.64	17.4	16.2
3	After 77 minutes.	18.16	7.28	34	17.08	15.5	14.8

Experiment 6—Concluded.

Time.	Ventilation rate.	Per cent Δ.
	liters per min.	
Day before.	4.4	
10.30 a.m.	4.6	
Mean before ether.	4.5	$\pm$ 0
11.05 a.m.	5.6	$+$ 22
11.24 "	4.1	$-$ 9
11.35 "	5.8	$+$ 29
11.11 "	8.4	$+$ 87
11.47 "	7.9	$+$ 76
11.55 "	10.6	$+$136
12.02 p.m.	9.6	$+$113
12.08 "	10.2	$+$127

BIBLIOGRAPHY.

Atkinson, H. V., and Ets, H. N., *J. Biol. Chem.*, 1922, lii, 5.

Austin, J. H., Cullen, G. E., Hastings, A. B., McLean, F. C., Peters, J. P., and Van Slyke, D. D., *J. Biol. Chem.*, 1922, in press.

Bohr, C., *Skand. Arch. Physiol.*, 1905, xvii, 104.

Caldwell, G. A., and Cleveland, M., *Surg., Gynec. and Obst.*, 1917, xxv, 23.

Carter, W. S., *Arch. Int. Med.*, 1920, xxvi, 319.

Collip, J. B., *Brit. J. Exp. Path.*, 1920, i, 282.

Cullen, G. E., *J. Biol. Chem.*, 1922, lii, 501.

Dale, H. H., and Evans, C. L., *J. Physiol.*, 1920-21, liv, 167.

Hasselbalch, K. A., *Biochem. Z.*, 1917, lxxviii, 112.

Hasselbalch, K. A., and Lundsgaard, C., *Skand. Arch. Physiol.*, 1912, xxvii, 13.

Henderson, Y., and Haggard, H. W., *J. Biol. Chem.*, 1918, xxxiii, 345.

Lewis, T., Cotton, T. F., Barcroft, J., Milroy, T. R., Dufton, J., and Parsons, T. R., *Brit. Med. J.*, 1916, ii, 517.

McLean, F. C., Murray, H. A., Jr., and Henderson, L. J., *Proc. Soc. Biol. and Med.*, 1919-20, xvii, 181.

Menten, M. L., and Crile, G. W., *Am. J. Physiol.*, 1915, xxxviii, 225.

Milroy, T. H., *Quart. J. Exp. Physiol.*, 1914-15, viii, 141.

Parsons, T. R., *J. Physiol.*, 1919-20, liii, 42.

Reimann, S. P., and Hartman, F. L., *Am. J. Physiol.*, 1919-20, l, 82.

Van Slyke, D. D., *Proc. Nat. Acad. Sc.*, 1921, a, vii, 229.

Van Slyke, D. D., *J. Biol. Chem.*, 1921, b, xlviii, 153.

Van Slyke, D. D., *J. Biol. Chem.*, 1922, a, lii, 525.

Van Slyke, D. D., *J. Biol. Chem.*, 1922, b, lii, 495.

Van Slyke, D. D., and Stadie, W. C., *J. Biol. Chem.*, 1921, xlix, 1.

Van Slyke, D. D., Austin, J. H., and Cullen, G. E., *Proc. Soc. Exp. Biol. and Med.*, 1919-20, xvii, 169.

STUDIES ON EXPERIMENTAL RICKETS.

XXI. AN EXPERIMENTAL DEMONSTRATION OF THE EXISTENCE OF A VITAMIN WHICH PROMOTES CALCIUM DEPOSITION.

By E. V. McCOLLUM, NINA SIMMONDS, and J. ERNESTINE BECKER.

(From the Department of Chemical Hygiene, School of Hygiene and Public Health, the Johns Hopkins University, Baltimore.)

and P. G. SHIPLEY.

(From the Department of Pediatrics, the Johns Hopkins University, Baltimore.)

With the discovery in 1913 that certain fats contain a substance or substances which are essential for growth, this class of foodstuffs assumed an importance in nutrition which had not been hitherto accorded it. At first the evidence of the existence of the substance which subsequently became known as fat-soluble A, or vitamin A, rested upon the failure of experimental animals to grow when the fats carrying this substance were lacking in the food, and the resumption of growth when such fats were administered. Later xerophthalmia of a certain type was recognized as a pathological condition which invariably results from specific starvation for fat-soluble A. We have recently, however, convinced ourselves that a similar ophthalmia may be the result of disturbance of the balance of inorganic elements in the diet. Up to the present time no definite evidence has been brought forward to show whether one or more than one substance is contained in those fats which contain fat-soluble A which gives them their unique biological value (1). The great activity in several laboratories in the study of the cause or causes of rickets and related conditions has, during the last 3 years, brought to light the fact that there is a rôle played by fats in the etiology of this disease. This observation causes us to appreciate further the importance of the fat moiety of the food supply, and emphasizes the necessity

293

of providing in the diet a regular and abundant supply of such fats as promote the normal development of the skeletal tissues.

Mellanby (2) was the first to associate the group of fats which, because of their content of fat-soluble A were frequently distinguished as "growth-promoting" fats with the prevention of rickets. His work focused attention upon the problem as to whether fat-soluble A is itself a substance essential for the normal growth of bone.

Mellanby (3) was so impressed with the power of butter fat to protect puppies against the abnormalities of bone growth that he stated:

"These facts of agreement from the point of view of physiological reaction seem to me strong evidence that the substance in fats stimulating the calcification of bone is the same as Fat-soluble A, i.e. the factor which stimulates growth in rats."

He further says:

"Rats as regards growth and puppies as regards rickets become more independent of the vitamin as they grow older. These facts are in favor of Fat-soluble A being also responsible for the anti-rachitic action of certain fats."

All of Mellanby's own data cannot be brought into line with this reasoning. For example, Dog 187 was fed bread, yeast, salt, orange juice, separated milk, and 10 cc. of cottonseed oil, but no meat. It developed rickets. Dog 305 had the same diet but with 10 gm. of meat   The bones of this animal are described as "practically normal" (3). Meat is essentially lacking in fat-soluble A. It does, however, contain phosphorus, and but little calcium, and serves to change the ratio between these elements in the diet. This, as has been pointed out, is of great importance in the etiology of rickets (4).

In his experiments Mellanby studied cod liver oil, butter fat, lard, suet, bacon fat, peanut oil, olive oil, coconut oil, linseed oil, babassu oil, rape seed oil, palm kernel oil, and hydrogenated fats. He states (3):

"Of the fats tested, cod-liver oil is the best. Suet and butter also have a potent influence on calcification. Lard is poor as compared with suet. Butter, heated and oxidized for four hours, loses some anti-rachitic action.

Cod liver oil similarly treated still has a strong anti-rachitic effect. The vegetable oils vary in their anti-rachitic action, the order of merit being somewhat as follows: pea-nut and coco-nut oils (best), rape-seed, cotton-seed, palm-kernel, olive, linseed, and babassu oils (worst). Hydrogenated fats are poor."

It will be seen from the foregoing quotations that the question as to whether fat-soluble A exerts an antirachitic effect, or whether such action is to be referred to some other principle which has in many cases at least, a similar distribution is still open.

In July, 1921 (5) we pointed out on the basis of experimental data that

"We have not found it possible to demonstrate experimentally a differ-ence between the effects of cod liver oil and butter fat when the content of the diet in calcium and phosphorus is near the optimum, the other elements in the diet being satisfactory."

We stated further that

"These results suggest the possibility that a dietary essential distinct from the anti-ophthalmic substance (fat-soluble A) may exist. If this is the case this would appear to be present in butter fat in small amounts, but to be very abundant in cod liver oil."

Again, in January, 1922 (6) we set forth evidence which was all but conclusive that there is a specific calcium-depositing substance. We stated

"The results of this series of experiments were so consistent and decisive that we can deduce no other conclusion than that cod liver oil contains in abundance some substance which is present in butter fat in but very slight amounts, and which exerts a directive influence on bone development and enables animals to develop with an inadequate supply of calcium much better than they could otherwise do. This substance is apparently dis-tinct from fat-soluble A, which is essential for growth and which is asso-ciated definitely with the prevention of ophthalmia (keratomalacia)."

Our own experience had convinced us that existing methods were incapable of differentiating beyond doubt between fat-soluble A and a special calcium-depositing substance should such exist. We therefore formulated a plan which involved a comparison of a selected list of fats in respect to three kinds of effects in nutrition. First, we tested cod liver oil, shark liver oil, butter fat, and several vegetable oils for potency in causing the cure of xerophthalmia

due to lack of fat-soluble A. Secondly, we made comparative tests of the same fats to determine their value in promoting growth in young rats which were restricted to a diet so low in calcium that satisfactory growth was not possible without the provision of some substance which would make for a greater efficiency in the utilization of calcium than that which could be effected in its absence. Thirdly, we further studied these same fats by means of our "line test" to discover their relative values for inducing the deposition of the line of calcium salts in rachitic bones. With the data which we have secured from these three distinct types of tests, we are now in a position to interpret accurately the results of much of the experimental data in the literature which is otherwise confusing.

EXPERIMENTAL PROCEDURE.

I. Test for Fat-Soluble A.

The diet used for testing the value of different fats for the cure of xerophthalmia had the following formula.

Lot 3392.

	per cent
Rolled oats............	...............40.0
Casein.......	.... 5.0
NaCl................ ...	.... 1.0
CaCO₃	.... 1.5
Dextrin...............................	.................52.5

This diet is essentially lacking in fat-soluble A. If 2 per cent of butter fat or cod liver oil is included in place of an equivalent amount of dextrin, xerophthalmia never develops, and the animals are able to grow.

In making the tests referred to in this paper young rats of 40 to 60 gm. weight were restricted to Diet 3392 until the puffiness of the eyelids was distinctly evident. At this point the eyes were frequently sealed shut while the rats slept, and were opened with difficulty on awakening. The malnutrition induced by this diet progresses rapidly to a fatal termination unless a suitable amount of fat-soluble A is provided at this stage. On the addition of sufficient fat containing fat-soluble A, at the time when the

edema of the eyelids is just becoming severe the swelling rapidly disappears, and the eyes return to a normal appearance within a few days. 2 per cent of cod liver oil, 3 per cent of shark liver oil, 3 per cent of burbot liver oil,[1] or 2 per cent of butter fat, were found to effect the prompt cure of incipient xerophthalmia under the conditions of our tests. Although we have data from other experiments which indicate that certain of the vegetable oils when fed liberally (8 to 20 per cent) from the beginning of the experiment tended to defer the onset of xerophthalmia, we have never found that such amounts of vegetable fats would cure the eye condition after it had once developed. It is, therefore, possible that there are traces of fat-soluble A in some vegetable fats. It should be specially noted here that 15 per cent of coconut oil did not cure or prevent xerophthalmia. We are convinced that a properly conducted *curative* test such as we have described is much more delicate than a *preventive* test can ever be made.

Hopkins (7) was the first to point out that oxidation destroys fat-soluble A. He showed that if oxygen is allowed to pass through heated butter fat the fat-soluble vitamin is readily destroyed. With this destruction the butter fat loses its power of inducing growth or of curing ophthalmia of dietary origin. Mellanby attempted (3) to make use of this means of destroying fat-soluble A in order to determine whether there is a distinct "anti-rachitic substance." He found butter fat of little value for protecting against rickets after it had been oxidized, whereas cod liver oil after the same treatment, *i.e.* heated to 120°C. for 4 hours while oxygen was passing through it, still protected his animals against rickets. He states:

"If it should happen that four hours' heating and oxidation at 120°C. also leaves a large amount of Fat-soluble A in the cod liver oil, it will go a long way, especially when considered together with the butter results, to clinch completely the identity of fat-soluble A and the anti-rachitic vitamine."

Mellanby used no method of testing for fat-soluble A as distinct from the calcium-depositing substance since he did not make use of the ophthalmia test for fat-soluble A.

[1] We are indebted to Miss Ethel Kalmbach of Sturgeon Bay, Wis., who kindly furnished us a large sample of carefully prepared oil from the livers of the burbot.

We have found that cod liver oil treated with a stream of air bubbles at the temperature of boiling water for 12 to 20 hours no longer contains sufficient fat-soluble A to relieve rats from xerophthalmia when administered to the extent of 2 per cent of the diet. Cod liver oil which had been oxidized 4 hours, when fed as 2 per cent of the diet, cures xerophthalmia. Untreated cod liver oil under these conditions invariably causes complete recovery within 5 days. Likewise, 2 per cent of fresh butter fat, under exactly comparable experimental conditions, effects the disappearance of ophthalmia within 5 to 10 days. These results are sufficient to serve as a basis of comparison for our present purpose of the relative values of cod liver oil and butter fat for the cure of ophthalmia. The significance of these results in connection with the problem of the existence of a special calcium-depositing vitamin will be discussed later.

II. Tests of Fats for Their Protective Power against the Effects of Deficient Calcium Supply.

In a former paper (5) we have described experiments which showed clearly that a diet may be so deficient in calcium as to prevent growth in young rats. Butter fat failed to protect the animals from the effects of calcium deficiency, whereas the same diet supplemented with cod liver oil may promote good growth. We have since refined the technique of this type of experiment for studying the influence of fats on calcium metabolism. For this purpose we now use the following diet.

Diet 2947.

	per cent
Whole wheat	25.0
Whole maize	19.5
Rice polished	9.5
Rolled oats	9.5
Whole milk powder	5.0
Peas	9.5
Navy beans	9.5
Casein	10.0
NaCl	1.0
Dextrin	1.5

The dietary properties of this food mixture have been carefully determined. The proteins are of very good quality and are abundant (about 23 per cent). Its content of water-soluble B is entirely adequate. It is somewhat deficient in fat-soluble A, but not sufficiently so as to induce xerophthalmia even if an animal were confined throughout life to it. Its inorganic content was such as to induce skeletal deformities. The diet was low in calcium, and not far from the optimum in phosphorus, and our problem was to see to what extent if any the different fats increase the efficiency of the animals in utilizing the small amount of calcium at their disposal.

This food mixture was fed with the following fats: cod liver oil 1.0 per cent; butter fat 10.0 per cent; shark liver oil 3.0 per cent; coconut oil 10.0 per cent; cottonseed oil 10.0 per cent; and olive oil 10.0 per cent.

An inspection of the charts shows that on this diet the animals cannot grow appreciably when the fat which is supplied them, is cottonseed or olive oil. When cod liver oil, shark liver oil, or butter fat is fed, growth proceeds in a fairly satisfactory manner. Coconut oil is the only one of the vegetable oils examined which increased the efficiency of the animals in utilizing their very low calcium supply. These data show clearly that there is a very remarkable property of certain fats which makes them of extraordinary importance in relation to calcium metabolism.

III. Tests of Fats for Their Power to Stimulate Healing in Rickets.

The data presented above relative to the values of several fats for relieving xerophthalmia, and for increasing the efficiency of the tissues in utilizing calcium when this element is present in very inadequate amounts in the diet, do not constitute a safe basis for deciding whether the effects in both types of experiments were due to fat-soluble A, or to two distinct substances.   The results of the experiments in which coconut oil was studied (Chart 4, Lot 3008) suggest strongly that this oil contains a substance which improves the utilization of calcium.   It has been pointed out above on the basis of experimental tests that coconut oil does not contain the substance, fat-soluble A, which

relieves xerophthalmia, since no improvement follows the inges-
tion of liberal amounts of this fat even in the incipient stages
of the eye disease. Experiments directed toward preventing
xerophthalmia by feeding coconut oil confirmed this view. We,
therefore, supplemented the data obtained by the above described
tests, with observations on the effects of the several fats on the
initiation of the healing process in rickets. For this purpose we
used the technique which we have called the "line test."

Diet No. 3143 employed in the "line test" consists of:

Diet 3143.

		per cent
Whole wheat kernel		33.0
Whole maize kernel		33.0
Gelatin		15.0
Wheat gluten		15.0
NaCl		1.0
CaCO$_3$		3.0

The details concerning the properties of this diet and its effects
on the bones have been given in another publication (8). It
is only necessary to state here that it contains a very inadequate
amount of fat-soluble A, but sufficient to prevent xerophthalmia
during the interval necessary to develop an exaggerated rickets
and to observe the incipient healing of the lesion. Although the
diet contains an *excessive* amount of calcium, no deposition of
calcium salts takes place.

A few words of explanation are necessary regarding the column
in Table I headed "Number of days in preparatory period."   In
order to carry out the "line test" satisfactorily the animals are
fed Diet 3143 until a pathological metaphysis has developed
satisfactorily. This state we have come to recognize by certain
peculiarities in the movements of the young rats which we use as
the test animals. The gait is unsteady and the hind quarters
waver from side to side. When they move off rapidly they hop,
usually favoring one hind leg. We have found it safe to rely on
young rats which exhibit this abnormality of movement to show
the histological picture which is essential for the conduct of the
test.

We have examined histologically the bones of a large number of
rats which have been kept on this diet for varying periods, and

TABLE I.

Kind of oil.	Per cent	Number of days in preparatory period	Number of days fat was given.	Number of animals.	Results.
Cod liver oil.	2	21	5	6	Healing rickets.
" " "	2	25	6	2	" "
" " "	2	28	11	3	" "
" " "	2	49	5	3	" "
" " "	2	49	5	3	" "
" " "	0.2	70	5	1	Severe rickets, a few specks of calcium in cartilage.
" " "	0.4	70	5	1	Severe rickets, no healing.
" " "	0.6	70	5	1	Beginning healing of rickets.
Cod liver oil (oxidized 4 hrs.)	2	25	6	3	Healing rickets.
Cod liver oil (oxidized 12 hrs.)	2	28	11	3	" "
Cod liver oil (oxidized 20 hrs.)	2	29	10	3	" "
Butter fat.	30	21	14	2	Beginning healing.
" "	30	32	11	4	" "
" "	15	17*	12	4	3 of these animals showed occasional specks of calcium in cartilage.
" "	15	20	20	1†	Beginning healing rickets.
Shark liver oil.	2	34	11	3	Marked healing.
Burbot liver oil.	2	30	6	2	Healing well on.
" " "	2	30	10	4	" " "
Coconut oil.	20	24	15	5	Very slight evidences of healing.
Maize oil.	20	34	15	5	Severe rickets, no healing.
Olive "	20	34	15	5	" " " "
Cottonseed oil.	20	29	14	5	" " " "
Sesame oil.	20	24	15	5	" " " "

* This preparatory period was too short. The metaphyses were narrow and irregular. It was possible to mistake calcified matrix which had existed before the onset of he disease as evidence of healing.

† This animal was badly deformed and feeble after being fed the butter fat for 20 days. There was no clinical evidence that the butter fat had been at all beneficial.

have found considerable difference in the length of time required to prepare them for the test for the calcium-depositing vitamin.   We have gained the impression that it takes their bones a decidedly longer time in summer than in winter to deviate from the normal histology in the manner desired.   Certainly they do not all respond in a certain number of days so that it is not possible to state accurately when they will be ready.   We can, however, rely with certainty on the peculiarities of movement described above as a criterion of the time when a substance to be tested should be administered.   The age of the animals is an important factor in the rate at which they may be prepared for the test. Rats weighing 55 to 60 gm. are as a rule, in fall, spring, and winter, ready in 28 to 35 days.

We have studied in this way the fats already discussed, and have included maize oil and sesame oil among the vegetable oils examined.   Coconut oil was found to be distinctly more effective as a calcium-depositing agent than any of the other vegetables tested.   It was, however, inferior to butter fat or any of the fish oils tested.

The several oils studied are listed in Table I.   The amounts fed, the number of days of administration, the length of the pre-liminary period, the number of animals tested, and the results of the tests are given.   Since starvation causes the deposition of calcium salts in the bones under the conditions of this test, we have invariably kept accurate records of food consumption in making these tests.   The food consumption was adequate in all cases (9).

An inspection of Table I shows that cod liver oil, shark liver oil, and burbot liver oil, were highly effective in moderate doses in causing the deposition of calcium in the bones of rachitic animals.   Butter fat is also effective when fed in large amounts (15 to 30 per cent), but it was necessary to extend the time of administration to 14 days in order to obtain even a faint calcifi-cation of the bones.

Coconut oil, when fed at 20 per cent of the diet, caused in 15 days the deposition of small amounts of calcium salts in the bones under the conditions of our test.   Maize oil, olive oil, cottonseed oil, and sesame oil, were likewise fed at 20 per cent of the diet for 14 to 15 days, but in no case was there any tendency to the de-position of calcium salts.

.   Samples of cod liver oil which had been oxidized for 4, 12, and 20 hours, respectively, were tested for their calcium-depositing properties.   Those oxidized 12 and 20 hours showed this potency in a degree apparently comparable with similar amounts of unoxidized samples, notwithstanding the fact that they had entirely lost their power to cure xerophthalmia.   The samples which had been oxidized for 4 hours still cured xerophthalmia when 2 per cent of the diet consisted of the oil.   They likewise gave a positive test for calcium-depositing power.

DISCUSSION OF RESULTS.

We have shown experimentally that cod liver oil oxidized for 12 to 20 hours does not cure xerophthalmia in rats.   It does, however, cause the deposition of calcium in the bones of young rats which are suffering from rickets.   This shows that oxidation destroys fat-soluble A without destroying another substance which plays an important rôle in bone growth.

Coconut oil is shown to be lacking in fat-soluble A, since it will neither prevent nor cure xerophthalmia.   This oil, on the other hand, contains a substance which stimulates the deposition of calcium salts in rickets in a manner similar to cod liver oil.   It is, like butter fat, far less effective from a quantitative standpoint.

Cod liver oil, shark liver oil, and burbot liver oil, are highly effective for curing xerophthalmia, for protecting the body against the effects of a deficiency of calcium, and for the deposition of lime salts in rachitic bones.

Certain vegetable fats, among which are cottonseed oil, maize oil, sesame oil, and olive oil, do not possess the property of curing xerophthalmia, nor do they raise the efficiency of the tissues in utilizing calcium when there is an inadequate provision, nor of initiating healing in rickets.

Butter fat contains the calcium-depositing factor but in much smaller amounts than the fish oils we have examined.   It is a much better source of fat-soluble A than of the substance which regulates calcium metabolism.

Our results are in harmony with those of Mellanby in that they show that coconut oil has an antirachitic effect.   They prove conclusively, however, that this effect is not due to the presence

of fat-soluble A in this fat. Mellanby did not appreciate the
importance of calcium and phosphorus in his experimental diets
as a factor in the causation of rickets. Many of his apparently
discordant results can, we believe, be accounted for on this basis
rather than on the content of his diets in the calcium-depositing
vitamin (3).

The question might be raised as to whether the effect of the
substance which is designated fat-soluble A in protecting the
functional activity of the eyes, is not one, and the calcium-de-
positing effect of certain oils which contain it, another physiologi-
cal effect of one and the same substance. A controversy involving
this principle has been carried on for many years over the identity
or non-identity of pepsin and rennin. It may be postulated that
the two properties which cod liver oil and certain other fats can
be shown to possess, are referable to two side chains on the same
molecule, and that in oxidation we have destroyed one and left
the other intact. It is, of course, not possible at present to prove
or disprove either of these views. The only evidence which
bears on the question is the observation that coconut oil which
had received no chemical treatment whatever, has been shown to
possess demonstrable calcium-depositing properties, whereas it
does not show a comparable antixerophthalmic effect. This
points to the two properties under discussion being due to distinct
substances.

The evidence set forth in this paper demonstrates that the
power of certain fats to initiate the healing of rickets depends on
the presence in them of a substance which is distinct from fat-
soluble A. These experiments clearly demonstrate the existence
of a fourth vitamin whose specific property, as far as we can tell
at present, is to regulate the metabolism of the bones.

CHART 1. Lots 2765 and 3006 were fed diets which were essentially
identical in their dietary properties except in so far as these were modified
by the added fats. Diet 2765 contained 1.0 per cent of cod liver oil and
Diet 3006 contained 10.0 per cent of a bleached cottonseed oil (Wesson oil).
The defects in these diets were limited, as far as we can definitely charac-
terize them at present, to a deficiency in calcium which was closely com-
parable in the two diets, and in such organic factors as certain oils (e.g. cod
liver oil or butter fat) can supply.

The difference in the well being of these two groups of rats, due entirely
to the qualities of the fats which they were fed, was most remarkable and

CHART 1, LOT 2765
Ration:

Wheat	25.0
Maize	20.0
Rice	9.5
Rolled oats	9.5
Peas	9.5
Navy beans	10.0
Casein	1.0
NaCl	1.0
Whole milk powder	5.0
Cod liver oil	1.0

LOT 3006
Ration:

Wheat	20.0
Maize	15.0
Rice	9.5
Rolled oats	9.5
Peas	10.0
Navy beans	10.0
Casein	10.0
NaCl	1.0
Whole milk powder	5.0
Cottonseed oil	10.0

GRAMS
360
320
280
240
200
160
120
80
40

Y = birth of young

Lot 3006

Lot 2765

2nd generation

< 4 >
Weeks

Y

CHART 2, LOT 2766

Ration:

Wheat	20.0
Maize	15.0
Rice	9.5
Rolled oats	10.0
Peas	10.0
Navy beans	10.0
Casein	1.0
NaCl	5.0
Whole milk powder	10.0
Butter fat	

Y = birth of young

2nd generation

Lot 2766

are illustrated by the curves in the chart. The group receiving the cod liver oil grew well and were fairly fertile and succeeded, notwithstanding the lack of calcium, in rearing most of their young. The group fed cottonseed oil, although they consumed about ten times as much fat, grew but little and failed early without having any young.

Lot 2765 received a suitable amount of both fat-soluble A and of the calcium-depositing vitamin, whereas Lot 3006 was essentially deprived of the calcium-depositing vitamin and secured but an inadequate amount of fat-soluble A in the seed products and milk powder which the diet contained. Lot 2765 was capable, because of the character of their diet, of utilizing effectively the small amount of calcium at their disposal, whereas Lot 3006 could not do this.

CHART 2. Lot 2766 had a diet like those described in Chart 1, but with 10 per cent of butter fat instead of cod liver oil or cottonseed oil. These animals grew fairly well but were decidedly inferior to those described in Chart 1, which received 1 per cent of cod liver oil. These records emphasize how very important it is to have in the diet certain fats possessing unique properties, when there is an unfavorable concentration of calcium in the food. Certain fats greatly protect the cells against their faulty chemical environment and enable them to utilize better than would otherwise be possible their very inadequate calcium supply. This, we are now in a position to assert, is due to the content in such fats of a special calcium-depositing vitamin which is often associated with fat-soluble A, but is distinct from it.

CHART 3. Lots 3012 and 3005 had the same food mixture discussed in Charts 1 and 2 except that the former had 3.0 per cent of shark liver oil and the latter 10 per cent of virgin olive oil. The contrast between the value of the fish oil on the one hand and the vegetable oil on the other in protecting the animals against the detrimental effects of a deficiency of calcium in the diet is very striking. Lot 3012 had a sufficient amount of both fat-soluble A and of the calcium-depositing vitamin. Lot 3005 had a suboptimal amount of the former, but very little of the latter, derived from the small amount of milk in its diet. The shark liver oil was not so effective as cod liver oil in raising the potential of the body cells so as to make them capable of utilizing an insufficient calcium supply, but it was distinctly better than butter fat for this purpose.

CHART 4. This chart is of special importance when its two groups of animals, Lots 3008 and 2947, are compared, and also these with Lot 3005, Chart 3, and Lot 3006, Chart 1.

Lot 2947 had the experimental diet previously discussed but without any added fats. On this they were able to grow but poorly, remaining undersized and infertile, and they deteriorated at an age when, on a better diet, they would still have been in possession of full vigor.

Lot 3008 is of special interest when compared with Lot 2947 which had the same diet without added fats and Lot 3005 (Chart 3) which had 10 per

CHART 3. LOT 3012

Ration:	
Wheat	20.0
Maize	22.0
Rice	9.5
Rolled oats	9.5
Peas	10.0
Navy beans	10.0
Casein	10.0
NaCl	1.0
Whole milk powder	5.0
Dark liver oil	3.0

LOT 3005

Ration:	
Wheat	20.0
Maize	16.0
Rice	9.5
Rolled oats	9.5
Peas	10.0
Navy beans	10.0
Casein	10.0
NaCl	1.0
Whole milk powder	5.0
Olive oil	10.0

GRAMS: 400, 360, 320, 280, 240, 200, 160, 120, 80, 40

4 Weeks

Lot 3012

Lot 3012 2nd generation

Lot 3005

CHART 4, LOT 3008

Ration:

Wheat	20.0
Maize	15.0
Rice	9.5
Rolled oats	9.5
Peas	10.0
Navy beans	10.0
Casein	10.0
NaCl	1.0
Whole milk powder	5.0
Coconut oil	10.0

LOT 2947

Ration:

Wheat	25.0
Maize	19.5
Rice	9.5
Rolled oats	9.5
Peas	9.5
Navy beans	9.5
Casein	10.0
NaCl	1.0
Dextrin	1.5
Whole milk powder	5.0

Y = birth of young

GRAMS: 280 240 200 160 120 80 40

< 4 >
Weeks

Lot 3008   Lot 2947

CHART 5, LOT 2441

Ration:

Wheat	25.0
Maize	19.5
Rice	9.5
Rolled oats	9.5
Peas	9.5
Navy beans	9.5
Steak (round)	10.0
Whole milk powder	5.0
NaCl	1.0
$CaCO_3$	1.5

GRAMS

320
280
240
200
160
120
80
40

2nd generation   3rd generation   4th generation

Y = birth of young

< 4 >
Weeks

♂
♀

310

cent of olive oil, and Lot 3006, Chart 1, which had 10 per cent of cotton-seed oil.

One striking feature about this series of curves is that Lot 3005 (Chart 3) was not quite so well nourished on its diet containing olive oil as was Lot 2947 which had the same diet with no fats added. This difference was not sufficiently pronounced to establish it as significant further than to show that olive oil certainly does not have the property of increasing the efficiency of the cells in functioning with an inadequate calcium supply.

The diet consumed by Lots 3005 and 2917 was the same as that of Lot 3006 (Chart 1) but modified in that cottonseed oil was included to the extent of 10 per cent. A comparison of these groups shows that the basal diet itself did not raise the efficiency of the cells in utilizing their insufficient calcium supply. Cottonseed oil and olive oil lack, therefore, a property which is possessed in high degree by cod liver oil, shark liver oil, burbot liver oil, and to a lesser extent, by butter fat. These fats of animal origin differ from the two plant oils mentioned in that the former exercise a pronounced protective action in directing the functioning of the anatomic elements of the osseous tissues, whereas the latter do not.

Of special interest is the record of Lot 3008, whose diet was the same as those just discussed except that it contained 10 per cent of coconut oil. With this diet there was an observable protective effect due to the coconut oil. There is, therefore, a slight difference between olive oil and cottonseed oil on the one hand, and coconut oil on the other, with respect to the special effect on the bone growth. The animals on this diet containing coconut oil were not so much protected by that oil as their growth curves would seem to indicate. They were very short bodied and stocky creatures with rough coats and presented rather a miserable appearance notwithstanding the fact that they grew at a slow rate to about two-thirds the normal adult size.

If we employ this diet without the coconut oil or other added fat and add 1.5 per cent of calcium carbonate, the animals thrive far better than they do with the calcium omitted and coconut oil included. See Chart 5.

It should be kept in mind in interpreting these records that the diets are not very faulty in any respect other than in a lack of calcium. The small content of milk powder containing some butter fat, and the small amount of fat-soluble A furnished by wheat and maize, gave the animals nearly enough of this principle to meet their minimal requirements. The butter fat contained in the milk powder supplemented the very feeble protective power of the coconut oil in respect to its effects on the bones due to the calcium-depositing vitamin. Any fat possessing in considerable degree the special property of increasing the efficiency of the cells in utilizing a low calcium supply would have exercised a much more marked beneficial effect on the general well being of the animals than did the coconut oil.

CHART 5. The records in this chart illustrate the fact that animals can undergo apparently normal development when furnished a very small amount of the calcium-depositing vitamin, provided the diet contains

somewhere near the optimal content of calcium and phosphorus. The adjustment of the calcium content of these diets at a very low level has enabled us to demonstrate the difference in the quality of fats in respect to their content of the calcium-depositing vitamin in a manner which would have been impossible if the diets had contained a more favorable concentration of calcium and phosphorus.

BIBLIOGRAPHY.

1. McCollum, E. V., Simmonds, N., Becker. J. E., and Shipley, P. G., An experimental demonstration of the existence of a vitamin which promotes calcium deposition, *Bull. Johns Hopkins Hosp.*, 1922, xxxiii. 229.

2. Mellanby, E., An experimental investigation on rickets, *Lancet*, 1919, i, 407; Accessory food factors (vitamines) in the feeding of infants, *Lancet*, 1920, i, 856.

3. Mellanby, E., Experimental rickets, *Med. Research Council, Special Rep. Series, No. 61,* 1921.

4. Sherman, H. C., and Pappenheimer, A. M., A dietetic production of rickets in rats and its prevention by an inorganic salt, *Proc. Soc. Exp. Biol. and Med.*, 1920–21, xviii, 193. Shipley, P. G., Park, E. A., McCollum, E. V., and Simmonds, N., Studies on experimental rickets. III. A pathological condition bearing fundamental resemblances to rickets of the human being resulting from diets low in phosphorus and fat-soluble A: The phosphate ion in its prevention, *Bull. Johns Hopkins Hosp.*, 1921, xxxii, 160. Sherman, H. C., and Pappenheimer, A. M. Experimental rickets in rats. I. A diet producing rickets in white rats, and its prevention by the addition of an inorganic salt, *J. Exp. Med.*, 1921, xxxiv, 189.

5. Shipley, P. G., Park, E. A., McCollum, E. V., and Simmonds, N., Studies on experimental rickets. VII. The relative effectiveness of cod liver oil as contrasted with butter fat for protecting the body against insufficient calcium in the presence of a normal phosphorus supply, *Am. J. Hyg.*, 1921, i, 512.

6. McCollum, E. V., Simmonds, N., Shipley, P. G., and Park, E. A., Studies on experimental rickets. XII. Is there a substance other than fat-soluble A associated with certain fats which plays an important rôle in bone development? *J. Biol. Chem.*, 1922, l, 5.

7. Hopkins, F. G., The effects of heat and aeration upon the fat-soluble vitamine, *Biochem. J.*, 1920, xiv, 725.

8. McCollum, E. V., Simmonds, N., Shipley, P. G., and Park, E. A., Studies on experimental rickets. VIII. The production of rickets by diets low in phosphorus and fat-soluble A, *J. Biol. Chem.*, 1921, xlvii, 507; Studies on experimental rickets. XVI. A delicate biological test for calcium-depositing substances, *J. Biol. Chem.*, 1922, li, 41.

9. McCollum, E. V., Simmonds, N., Shipley, P. G., and Park, E. A., Studies on experimental rickets. XV. The effect of starvation on the healing of rickets, *Bull. Johns Hopkins Hosp.*, 1922, xxxiii, 31.

ON A TYPE OF OPHTHALMIA CAUSED BY UNSATISFACTORY RELATIONS IN THE INORGANIC PORTION OF THE DIET. AN OPHTHALMIA NOT DUE TO STARVATION FOR FAT-SOLUBLE A, AND NOT CURABLE BY ITS ADMINISTRATION.

By E. V. McCOLLUM, NINA SIMMONDS, and J. ERNESTINE BECKER.

(From the Loboratory of Chemical Hygiene, School of Hygiene and Public Health, Johns Hopkins University, Baltimore.)

All investigators appear now to be convinced that a deficiency of fat-soluble A in the diet will, if pronounced and the deprivation continued over a sufficient interval, cause the development of a type of ophthalmia. The ophthalmia induced in the rat by lack of fat-soluble A we know, from the studies of Mori in this laboratory, involves a xerosis of the conjunctivæ, and is accurately described as xerophthalmia.[1] This disease is now regarded as due to specific starvation for fat-soluble A, which is found abundantly in certain fats of animal origin. As pointed out by Osborne and Mendel, fat-soluble A is exceptionally abundant in cod liver oil.[2] The appearance of edema of the eyelids, and of a sticky exudate in the eyes, the development of corneal ulcers, and the appearance of hypopyon, followed by a xerosis of the conjunctivæ, are now generally regarded as evidence of the absence or paucity of fat-soluble A in the food supply.

During the last few years we have occasionally observed cases of "sore eyes" in rats which were provided with an abundance of fat-soluble A. We were at a loss to explain this occurrence except through possible infection of the eyes by bacteria. We felt that if this condition had been due to infection resulting from the

[1] Mori, S., The primary changes in the eyes of rats which result from deficiency of fat-soluble A in the diet, *J. Am. Med. Assn.*, 1922, lxxix, 197.

[2] Osborne, T. B., and Mendel, L. B., The influence of cod liver oil and some other fats on growth, *J. Biol. Chem.*, 1914, xvii, 401.

breaking down of the natural barriers of defense it should have occurred among our enfeebled animals irrespective of the nature of the defects in their diets which caused their enfeeblement. This has not been the case. It has been confined to experimental

TABLE 1.

Percentage Composition of Diets.

Ration No.	Wheat germ.	Wheat gluten.	Egg albumin.	Gelatin.	Casein.	Dextrin.	Agar-agar.	Butter fat.	Salt mixture. No.	Salt mixture. Amount.	CaCO₃	NaCl	KH₂PO₄	NaH₂PO₄	KCl	MgO
	gm.	gm.	gm	gm.	gm.	gm.	gm.	gm.		gm.	gm.	gm.	gm.	gm.	gm.	gm.
3407	3*	15†		15		56.96‡	2	2	XXI	3.9	1.5			0.64		
3408	3*	5†		5	5.0	72.6‡	2	2	XXI	3.9	1.5					
3369	3	12	10	10		52.2	2	5	XX	4.1			1.7			
3399	3				18.0	68.0	2	5	185	3.7						0.3
3400	3	12	10	10		52.4	2	5	XXI	3.9			1.7			
3401	3*	12†	10§	10		54.3‡	2	2	XXI	3.9	1.5			1.3		
3402	3				25.0	59.4	2	5	XXI	3.9			1.7			
3417	3*				31.1	59.4‡	0	2	None.		3.0	1.0			0.5	
3370	3*				25.0	59.2	2	5	XX	4.1			1.7			
3366	3*	12†	10§	10		56.2‡	0	2	XX	4.1	1.5		1.2			
2074						16.8‖	0	3	IX	5.2						
3015	4				18.0	65.2	2	10	XII	0.8						
3369-A	3	12	10	10		54.3	2	5	185	3.7						
3402-A	3				25.0	59.6	2	5	185	3.7			1.7			
3305-A	4				18.0	69.3	2	5	185	3.7						
3418	3*				29.7	62.86‡	0	2	None.		0.093	1.0		0.85	0.5	
3442	3*				20.0	70.5‡	0	2	"		3.0	1.0			0.5	
2946	4				18.0	59.3	2	10	185	3.7	3.0					
3003	4				18.0	63.9	2	10	XI	2.1						

* Wheat germ extracted with ether and then with chloroform.

† Wheat gluten purified. See text.

‡ Dextrin made from specially purified starch.

§ Egg albumin purified. See text.

‖ This diet contained: polished rice, 75.0 gm.; Salt Mixture IX, 5.2 gm.; butter fat, 3.0 gm.; dextrin, 16.8 gm. The latter carried the alcoholic extract of 10 gm. of wheat germ.

groups employed in studying the effects of salt mixtures differing widely in composition. It may develop in animals which are growing fairly rapidly and present a moderately well nourished appearance. As soon as the eye disease appears the rats, as a rule, fail rapidly unless a suitable change is made in the diet.

TABLE II.

Composition of Salt Mixtures in Terms of Salts.

No. of salt mixture	NaCl	MgSO₄, anhydrous.	NaH₂PO₄ + H₂O	K₂HPO₄	CaH₄(PO₄)₂ + H₂O	Ferric lactate.	Calcium lactate (5H₂O)	KCl	K₂CO₃	CaHPO₄	CaCO₃	MgO	NaHCO₃	FeSO₄ + 7H₂O
	$gm.$	$gm.$	$gm.$	$gm.$	$gm.$	$gm.$	$gm.$	$gm.$	$gm.$	$gm.$	$gm.$	$gm.$	$gm.$	$gm.$
185	0.173	0.266	0.347	0.954	0.540	0.118	1.300			1.500				
IX	2.000							0.500			0.500	0.500		0.200
XX	0.500							1.000			1.500	0.200	0.700	0.200
XII	0.068							0.155	0.481		0.025	0.058		
XXI	0.500							1.000			1.500		0.700	0.200
XIII	0.246							0.193	0.835		0.177	0.122		
XI	0.319	0.266		1.429		0.111								

A Type of Ophthalmia

TABLE III

Composition of Salt Mixtures in Terms of Elements.

No. of salt mixture.	Percentage of salts in diet	K	Na	Ca	Mg	S	Cl	P	Fe	Employed in Diet No.
	per cent	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	
185	3 7	0 4280	0 1258	0 2742	0 0532	0 1003	0 1049	0 3803	0 0229	3399
IX	5 2	0 262	0 7870	0 5490	0 300	0 023	1 4400	0 2700	0 040	2074
XX	4 1	0 524	0 3890	0 600	0 120	0 023	0 7790	0 0000	0 040	3369
XII	0 8	0 3530	0 0660	0 010	0 035	0 137	0 115	0 153	0 000	3015
XXI	3 9	0 524	0 3890	0 600	0 000	0 023	0 7790	0 0000	0 040	3407
XIII	1 6	0 564	0 097	0 071	0 073	0 137	0 2410	0 153	0 000	3018
XI	2 1	0 253	0 126	0 000	0 053	0 071	0 194	0 641	0 023	3003

TABLE IV.

Inorganic Elements in Entire Rations per 100 Gm.

Ration No.	K	Na	Ca	Mg	S	Cl	P	Remarks.
	gm.	gm.	gm.	gm.	gm.	gm.	gm.	
3407	0 533	0 517	1 202	0 010	0 173	0 781	0 217	Sore eyes.
3408	0 533	0 411	0 202	0 010	0 121	0 781	0 088	" "
3369	1 142	0 523	0 620	0 143	0 167	0 920	0 463	Very sore eyes.
3399	0 428	0 126	0 274	0 233	0 237	0 105	0 533	Eyes normal.
3400	1 142	0 522	0 620	0 023	0 167	0 920	0 463	Sore eyes.
3401	0 533	0 623	1 202	0 010	0 153	0 781	0 359	" "
3402	1 021	0 411	0 602	0 010	0 223	0 781	0 633	" "
3417	0 271	0 416	1 202	0 010	0 246	0 846	0 298	" "
3370	1 021	0 411	0 602	0 130	0 223	0 781	0 633	" "
3366	0 877	0 411	0 602	0 730	0 153	0 781	0 345	" "
2074	0 289	0 809	0 555	0 319	0 076	1 477	0 340	" "
3015	0 353	0 066	0 010	0 035	0 137	0 155	0 153	Eyes normal.
3369-A	0 559	0 259	0 294	0 076	0 244	0 246	0 456	Recovery from sore eyes.
3402-A	0 925	0 148	0 276	0 063	0 300	0 107	1 013	Recovery from sore eyes.
3305-A	0 440	0 155	0 277	0 066	0 250	0 108	0 576	Eyes remain normal. Rats recover from ophthalmia on this diet.
3418	0 271	0 558	0 039	0 010	0 236	0 846	0 477	Sore eyes.
3442	0 271	0 416	1 202	0 010	0 162	0 846	0 203	" "
2946	0 440	0 155	1 477	0 066	0 250	0 108	0 576	Eyes normal.
3003	0 265	0 155	0 003	0 066	0 084	0 197	0 837	" "

From a study of our data involving the observation of rats on numerous types of diets we have come to the conclusion that there is a second type of ophthalmia produced by faulty diet. This condition may or may not be identical in its finer details with that which results from vitamin A starvation. The second type of

TABLE V.

Showing Types of Salt Supply Which Induce Ophthalmia and Others on Which Recovery Occurs.

Ration No.	K	Na	Ca	Mg	S	Cl	P	Remarks
	gm.	gm	gm.	gm.	gm.	gm.	gm.	
3369	1 142	0.523	0 620	0 143	0 167	0 920	0 463	Eyes very sore in 60 to 75 days.
3369-A	0 559	0 259	0 294	0 076	0 244	0 246	0 456	Animals which developed ophthalmia on Diet 3369 recovered promptly on Diet 3369-A.
3402	1 021	0 411	0 602	0 010	0 223	0 781	0 633	Eyes sore in 60 to 70 days.
3402-A	0 925	0 148	0 276	0 063	0 300	0 107	1 013	Animals which developed ophthalmia on Diet 3402, recover on Diet 3402-A.
3305-A	0 440	0 155	0 277	0 066	0 250	0 108	0 576	Recovery takes place on this diet.
3399	0 428	0 126	0 274	0 233	0 237	0 105	0 533	Notwithstanding the high magnesium content of this diet the eyes remain normal.*
2946	0 440	0.155	1 477	0 066	0 250	0 108	0 576	Eyes normal, notwithstanding the high calcium content.

* In our studies of the nutritive properties of certain plant seeds, we have added as much as 1 per cent of magnesium oxide to the diet without any eye symptoms or other pronounced ill effects appearing over periods covering 7 to 8 months.

ophthalmia we believe to be caused by an unfavorable relationship between certain inorganic elements in the food of the animals. As far as one can tell by an inspection of the external appearance of the eyes of rats which are suffering from the ophthalmia due

to an excessive or an unfavorable supply of mineral elements, the condition is indistinguishable from the ophthalmia resulting from lack of fat-soluble A.

Although we have accumulated experimental data which are very convincing, we do not regard the etiology of this form of ophthalmia as completely elucidated. Considerable time must elapse before the completion of our study of this problem. Since there is now great activity in a number of laboratories in the study of the distribution and properties of fat-soluble A, we report our more significant results because of the danger that confusion may result from the feeding of diets containing even liberal amounts of fat-soluble A, but otherwise so constituted as to lead to the development of the ophthalmia which we are now discussing, which has no connection with vitamin A deficiency.

DISCUSSION OF TABLES.

In Table IV the compositions of nineteen diets are shown. Some of these, as will be seen in the column under "Remarks," induced ophthalmia, whereas others did not. Certain of the diets were composed of highly purified food substances, whereas some contained wheat gluten and egg albumin which had not been treated so as to remove their non-protein constituents. The salt mixtures employed in these diets differed widely in composition. All diets contained fat-soluble A, the amount varying from the minimum on which good growth can be obtained to very liberal quantities of this vitamin. The butter fat was found in control experiments to contain apparently the usual content of fat-soluble A.

In Table II the compositions of the salt mixtures employed are given in amounts of the salts used in their preparation.

In Table III the compositions of the salt mixtures are given in terms of their content of the elements of physiological significance.

Table IV shows the amounts of mineral elements in certain diets on which rats develop ophthalmia notwithstanding the presence of such amounts of fats carrying fat-soluble A as will not only prevent xerophthalmia of the vitamin deficiency type, but will promote growth and well being over a long period. The salt composition of certain diets is shown on which the eyes of animals remain indefinitely in a normal. condition. Others are

tabulated on which ophthalmia developed, and still others to
which the rats were transferred, which led to prompt recovery.

Whenever the new type of ophthalmia developed the animals
were receiving what may be regarded as somewhat excessive
amounts of total mineral nutrients.[3] As will be seen from Table IV
the one element which was constantly present in high concentra-
tion was chlorine.   We have not yet tested diets which fulfill this
condition, but which were low in their sodium content.   We may
contrast on the one hand Lots 3407, 3408, 3369, 3400, 3401, 3402,
3417, 3370, 3366, 2074, 3418, and 3442, all of which developed the
eye condition, with Lots 3399, 3015, 3369-A, 3402-A, 3305-A,
2946, and 3003, which did not.   At first thought those which
received Salt Mixture 185 (Lots 3399, 3369-A, 3402-A, 3305-A,
and 2946) may appear to form exceptions to this statement.
An inspection of Table II will show, however, that the 3.7 gm. of
this salt mixture which was contained in each 100 gm. of food,
contained 1.30 gm. of calcium lactate, a salt containing 5 mole-
cules of water and but 14.48 per cent of calcium.   If the calcium
in this mixture had been added in the form of carbonate the total
amount of the salt mixture in 100 gm. of the food would have
amounted to but 2.87 per cent of the diet.   Salt Mixture 185
does not induce ophthalmia even when fed supplemented with
3.0 per cent of calcium carbonate (Diet 2946), or with potassium
phosphate 1.7 per cent (Diet 3402-A), or with magnesium oxide
0.3 per cent (Diet 3399).   These experiments suggest, therefore,
that excessive calcium, potassium, or magnesium is not the cause
of this ophthalmia.

The most significant data presented in the tables are brought
together in Table V.   Diet 3369 was very high in potassium, and
contained somewhat over what we have regarded as a satisfactory
amount of sodium, and about the optimal calcium and phosphorus
contents, but was very high in chlorine.   This diet has in our
experience invariably induced ophthalmia in 60 to 75 days.   The
same may be said regarding Diet 3402.

[3] When young rats are restricted to a salt-free diet they also develop
an ophthalmia which is apparently similar to that described here as the
result of excessive chlorine ingestion (Na ?).   The phenomenon is, there-
fore, not entirely specific, but involves certain disturbances in the content
of certain inorganic ions in the body fluids.   This problem is receiving
further study.

Animals which had developed the eye disease on Diet 3369 recovered promptly when Salt Mixture XX, together with 1.7 per cent of KH_2PO_4 was replaced by 3.7 per cent of Salt Mixture 185. This change reduced the content of the diet in all elements other than sulfur, which was increased somewhat, and phosphorus, which remained essentially unchanged. Animals fed Diet 3402 developed ophthalmia and recovered promptly when changed to Diet 3402-A, which differed only in the salt mixture added. The cure resulted, although the potassium content of the diet was nearly doubled. The sodium, calcium, and chlorine were reduced significantly.

Rats which had developed ophthalmia were also cured by transferring them to Diet 3395-A, which contained only 3.7 per cent of Salt Mixture 185. In this case we brought about a general reduction of mineral elements in the diet.

Diet 3399 contained 3.7 per cent of Salt Mixture 185, together with 0.3 per cent of magnesium oxide. The eyes remained entirely normal. In other experiments we have fed 1.0 per cent of magnesium oxide without causing ophthalmia.[4]

Diet 2946 contained 3.7 per cent of Salt Mixture 185, together with 3.0 per cent of calcium carbonate. The diet contained an excessive amount of calcium. The eyes of the animals on this diet remained normal.

We have repeatedly observed that rats fed diets which are optimal in composition with respect to all factors other than fat-soluble A, which is either lacking or nearly so, do not all develop xerophthalmia in the same length of time. It has been our experience that a deficiency of fat-soluble A does not cause xerophthalmia so quickly when the diet is optimal in respect to all other factors as it does when one or another factor other than vitamin A is of poor quality. In certain cases we have found the protein in the diet to exert a protective action in this respect. It may, therefore, be found in further work that a similar protective action will be exerted by other dietary factors in connection with the salt type of ophthalmia.

CONCLUSIONS.

From the data which we have discussed it is evident that the one constant factor which operated in the experiments in which

[4] McCollum, E. V., and Simmonds, N., Unpublished data.

ophthalmia was induced was a high content of chlorine. It is possible, however, that a high sodium content in the diet may contribute to cause this pathological condition. We suggest provisionally, therefore, that these are the etiological factors involved in inducing an eye condition which may be easily confused with the xerophthalmia due to lack of fat-soluble A. It may, of course, be found that the provision of excessive amounts of certain other inorganic elements may intensify the effect of chlorine, or sodium, or both, in hastening the onset of the eye disease. The whole question of the interrelation of salt effects in mammalian nutrition is still little understood.

Method of Purification of Egg Albumin.

1 pound of commercial powdered egg albumin (Merck) was placed in 10 liters of distilled water, and stirred occasionally until solution was complete. It was then heated over asbestos, and when the temperature had reached 80°C. acetic acid was added in amount sufficient to cause complete coagulation. After standing until the solution had cooled, the water was drained off on cheese-cloth, the coagulum returned to the vessel, and again brought to a boil with water acidulated with acetic acid. This process was repeated four times. Finally the albumin was drained on cheese-cloth, and dried in a current of warm air. It was then ground to a powder. This treatment is sufficient to remove practically all of the phosphorus, chlorine, and all the basic elements, from egg albumin.

Wheat Gluten.

500 gm. of commercial wheat gluten were treated with 5 liters of water to which acetic acid had been added to make a solution 0.2 of 1 per cent. The gluten was kept in the water and stirred at frequent intervals. At the end of the laboratory day the gluten was allowed to settle, the supernatant liquid removed, and replaced with a fresh solution. The treatment was continued during 6 days. The gluten was finally strained off on muslin and dried in a current of air. Wheat gluten treated in this way is almost free from phosphorus.

Purification of Starch.

5 pounds of commercial corn-starch were treated with 7 liters of 0.1 per cent hydrochloric acid. The starch was stirred at frequent intervals during the working day. The washing of the starch with 0.1 per cent hydrochloric acid was continued during 6 days. The starch was then washed with water, and the wet material acidified with citric acid and placed in a porcelain dish in an autoclave and heated at 18 pounds pressure for 1 hour to dextrinize it. The dextrin was then dried with a current of air.

CREATININE AND CREATINE IN MUSCLE EXTRACTS.

III. CONCERNING THE PRESENCE OF ENZYMES IN MUSCLE TISSUE WHICH HAVE CREATINE AND CREATININE AS THEIR SUBSTRATES.

By FREDERICK S. HAMMETT.

(*From The Wistar Institute of Anatomy and Biology, Philadelphia.*)

The determination of the presence or absence in muscle tissue of enzymes which affect the formation or destruction of creatine and creatinine and the transformation of creatine to creatinine is obviously of importance in connection with the problem of urinary creatinine.

Gottlieb and Stangassinger (1) reported results which they considered justified the belief of the presence of such enzymes in muscle tissue. Their findings were confirmed by other workers. Yet the negative results of Voegtlin and Towles (2) and others and the fact that the earlier methods of analysis were unreliable, seemed to justify a reexamination of the question.

Both Myers and Fine (3) and Hammett (4), using more exact methods of analysis, observed that when muscle extracts are aseptically incubated at body temperature there takes place in them an increase in creatinine at the expense of the creatine. This would seem to show that the process is enzymatic, were it not for the fact that the same change occurs in pure solutions of creatine in water, although at a slower rate.

In the experiments to be recorded in this paper the extracts which were used were made from the striated muscles of mature albino rats of both sexes. The muscle tissue was ground up in a meat chopper, macerated with fine sand and an equal weight of Tyrode's solution, and 10 cc. of toluene were added. The juice was then squeezed from the mixture by a filter press. The resultant extract was then diluted with an equal volume of Tyrode's solution, unless otherwise noted, and 5 cc. portions were

TABLE I

Effect of Dialysis on the Formation of Creatinine from Creatine in Muscle Extracts When Incubated at 38°C.

Series		Fresh extract	Un-dialyzed extract	After 24 hours incubation and dialysis				After 24 hours incubation when dialyzed extract and dialysate are incubated separately		
			A	Dialyzed extract B	B/A	Dialysate C	C/B	Dialyzed extract B'	Dialysate C'	C'/B'
		mg	*mg*	*mg*	*per cent*	*mg*	*per cent*	*mg*	*mg*	*per cent*
I	Creatinine.	0.070	0.171	0.172	0.4	0.178	3.5	0.263	0.220	−12.9
	Total creatinine.	5.97	5.76	5.77	0.2	5.90	2.3	5.18	5.54	0.5
II	Creatinine.	0.078	0.145	0.115	0.0	0.148	2.1	0.314	0.301	−3.2
	Total creatinine.	5.18	5.18	5.55	1.3	5.55	0.0	5.21	5.20	0.2
III	Creatinine.	0.083	0.175	0.171	−0.6	0.174	0.0	0.216	0.168	−22.2
	Total creatinine.	5.23	5.19	5.20	0.2	5.23	0.6	3.28	5.11	55.8
IV	Creatinine.	0.056	0.144	0.141	−2.1	0.154	9.2	0.355	0.292	−17.7
	Total creatinine	4.49	4.22	3.31	−21.6	5.01	52.3	5.98	6.04	1.0
V	Creatinine.	0.069	0.186	0.185	−0.5	0.186	0.5	0.513	0.356	−30.6
	Total creatinine.	5.95	6.02	6.05	0.5			6.00	5.95	0.8
VI	Creatinine.			0.216		0.205	−5.1			
	Total creatinine			6.76		6.72	−0.6			

VII	Creatinine.	0.182	0.182	0.0	0.400	0.312	−22.0
	Total creatinine.	6.69	6.72	0.4	5.96	6.00	0.7
VIII	Creatinine.	0.148	0.147	−0.7	0.229	0.213	− 7.0
	Total creatinine	4.79	4.79	0.0	4.80	4.80	0.0
IX	Creatinine.	0.154	0.154	0.0	0.366	0.352	− 3.8
	Total creatinine.	5.68	5.71	0.5			
X	Creatinine.	0.200	0.212	6.0	0.388	0.372	− 4.1
	Total creatinine.	5.75	5.80	0.9	5.80	5.73	− 1.2

invariably used for the analyses. The methods of analysis were the same as those previously described (4, 5). The temperature of the incubator was maintained at 38°. The extracts were protected from microorganisms by toluene. All the reported values for creatinine and total creatinine are averages of duplicate determinations. Those for creatinine represent the so called "preformed creatinine;" those for total creatinine, the preformed creatinine plus the creatine as creatinine.

An attempt was first made to separate the accelerating agent from the muscle extracts by dialysis. Samples of extract were dialyzed for 24 hours in the incubator, either in parchment or collodion thimbles, against an equal volume of Tyrode's solution. An equal amount of phosphate buffer mixture was added to each. This gave a pH between 6.9 and 7.0, determined colorimetrically. After the preliminary period of dialysis, the dialysate and the dialyzed extract were separated and aliquots incubated for a second 24 hours. Table I gives the results of the analyses made at the various periods.

It is seen that the amount of creatinine formed in the extract is the same whether the extract is diluted 1:1 before incubation or whether it is dialyzed against an equal volume of the diluent during incubation. The almost uniform appearance of equal concentrations of creatinine and total creatinine in the dialyzed extract and the dialysate after 24 hours incubation shows the easy diffusibility of these compounds. The results of the second 24 hour period of incubation are therefore strictly comparable.

It appears from this series of experiments as if the muscle extract as such provides a more favorable milieu for the transformation of creatine to creatinine than does its dialysate. But since the creatinine increase in the former is only 13.7 per cent greater than in the latter, the evidence for an enzyme participation is rather weak; unless it be believed that the enzyme, if present, is dialyzable.

Since no change in total creatinine occurred in these experiments or those to follow, it is evident that the increase in creatinine is at the expense of the creatine.

These differences in creatinine might be due to differences in hydrion concentration between extract and dialysate which Loeb (6) has shown to exist in similar experiments, and from the fact

that I have shown such differences result in differences in rate of creatinine formation (4).

During experiments which were made in the study of the course of the reaction, creatine-creatinine, it was noted that the degree of dispersion of the material in the colloid state[1] decreased with time. Advantage was taken of this fact to effect a partial separation of the colloids from the extract by centrifugation after 4 hours incubation. The centrifuged extracts were then incubated further, and the rate of creatinine formation in them compared with uncentrifuged extracts. The results of this series are given in Table II. The values indicate that the partial removal of colloids has slightly retarded creatinine formation. This confirms the findings of the dialysis experiments. The degree of difference is too slight to allow the conclusion that enzyme has been pulled out of the extract by adsorption.

Attempts were next made to destroy any enzyme which might be present by NaF, $HgCl_2$, and KCN. The first of these did not influence the reaction at all. The others interfered with the colorimetric procedure and were consequently useless.

Studies were now made of the rate of creatinine formation. 5 cc. lots of muscle extract were placed in test-tubes and put in the incubator. Creatinine and total creatinine were determined in the fresh extract and the incubated samples at 2 hour intervals for the ensuing 24 to 30 hours. The results of two such series are plotted on Chart 1. They show that the rate of creatinine formation is more or less periodic. Phases of acceleration, simulating the course of an autocatalyzed reaction are abruptly cut short, and then the process is repeated. Similar periodic changes in rate of reaction have been observed by Bredig and Weinmayr (7) and Bray (8).

It was noticed during the first of these experiments that marked changes were taking place in the degree of dispersion of the colloids in the muscle extracts. There is first a gel stage, and then flocculation sets in. These changes occur gradually. Similar changes in colloid equilibrium with time have been noted by du Noüy (9) in blood serum and by Davis, Oakes, and Browne (10)

---

[1] From now on the term "colloids" will be used for the sake of brevity instead of "materials in the colloid state."

TABLE II.

Effect of Partial Removal of Colloid Material from Muscle Extracts by Centrifugation on the Formation of Creatinine from Creatine during Incubation at 38°C.

Series	I		II		III		IV	
		A B		A/B		A/B		A/B
	mg.	per cent	mg.	per cent	mg.	per cent	mg.	per cent
Initial creatinine.	0.201		0.059		0.074		0.067	
Creatinine after 2 hrs. Untreated extract A .... Centrifuged extract B.......			0.103 0.094	9.6	0.130 0.117	11.1	0.107 0.095	12.6
Total creatinine, initial......	4.89		4.50		6.13		4.78	
Total creatinine, 2 hrs. Untreated extract A......... Centrifuged extract B.......			4.47 4.47	0.0	6.03 6.13	−1.6	4.76 4.78	−0.4
Creatinine after 24 hrs. Untreated extract A......... Centrifuged extract B.......	0.304 0.295	3.1	0.187 0.176	6.3	0.306 0.295	3.7	0.218 0.193	13.0
Total creatinine, 24 hrs. Untreated extract A .... Centrifuged extract B.......	4.91 4.96	−1.0	4.50 4.47	−0.7	6.03 6.08	−0.8	4.76	
Creatinine after 48 hrs. Untreated extract A......... Centrifuged extract B.......	0.411 0.408	0.7	0.374 0.338	10.7			0.331 0.336	−1.5
Total creatinine, 48 hrs. Untreated extract A......... Centrifuged extract B.......	4.89 4.91	−0.4	4.47 4.47	0.0			4.76 4.76	0.0
Creatinine after 72 hrs. Untreated extract A......... Centrifuged extract B.......			0.515 0.490	5.1			0.485 0.484	0.2
Total creatinine, 72 hrs. Untreated extract A......... Centrifuged extract B.......			4.47 4.50	0.7			4.80	
Creatinine after 100 hrs. Untreated extract A......... Centrifuged extract B.......			0.617 0.620	−0.5				
Total creatinine, 100 hrs. Untreated extract A......... Centrifuged extract B.......			4.47					

CHART 1. Showing periodicity in rate of creatinine formation during incubation under toluene at 38° at 2 hour intervals for 24 and 30 hours. Changes in total creatinine are also given.

329

in gelatin solutions.   They are significant of fundamental phenomena of colloid-containing solutions.

This observation raised the suspicion that the changes in rate of creatinine formation might be due to the changes in the colloid state of the extract. A study of the amount of material precipitated by uniform centrifugation at 2 hour intervals during incu-

TABLE III.

Showing Changes in Degree of Dispersion of the Colloid System in Muscle Extracts during Incubation at 38°C, as Determined by Centrifugation.

Time in hours.	I	II	III	IV	V
	cc.	cc	cc.	cc.	cc.
Fresh.	0.07	0.03	0.02	0.04	0.02
1	0.08	0.07	0.08	0.04	0.02
2	0.21	0.16	0.19	0.07	0.07
4	0.40	0.45	0.30	0.35	0.30
6	0.40	0.40	0.30		0.31
8	0.37	0.42	0.30	0.35	0.40
10	0.41	0.40		0.34	0.39
12	0.41	0.39	0.30	0.40	0.39
14	0.37	0.49	0.30		0.39
16	0.40	0.50			
18	0.45	0.50	0.30		
20	0.43	0.50	0.33		
22	0.41	0.52	0.37	0.39	
24	0.50	0.52	0.31		0.41
26			0.33		
28			0.31		
30		0.48			
48	0.52	0.50	0.33		0.49
72	0.68	0.47			0.49
96	0.68	0.53	0.33		
120	0.71		0.38		
168	0.70	0.63			

bation was made, using 5 cc. lots of extract. The results are given in Table III. They show that changes take place in the amount of precipitable matter of muscle extracts during incubation. It is evident that in the first 24 hour period the number and extent of these changes are greater than in later periods. The equilibrium which are established after each change tends to be fairly stable for increasing periods. These facts can be correlated

with the changes in rate of creatinine formation since the periodicity is only observed during the first 24 hours. Usually by 30 hours the course of the curve becomes regular. Moreover, it can be seen from Chart 1 that the early changes in rate of creatinine formation are in short periods, and that the periods tend to lengthen with time. A third correlation which is suggestive, is that in general the points where the changes in amounts of precipitable material occur are associated with the points where abrupt changes are taking place in rate of creatinine formation. Further proof that the hypothesis expressed above is well founded was obtained when the muscle extracts were deproteinized and then incubated.

Attempts at deproteinization with animal charcoal were unsatisfactory because of adsorption of creatinine and creatine. The writer, therefore, decided to attempt removal of the colloids from the extract by boiling, because it was hoped that a study of the creatinine formation in boiled extract would also show whether the reaction is catalyzed by an enzyme or not.

400 cc. of undiluted extract were prepared and divided into two lots of 200 cc. each. To one lot there were added 200 cc. of Tyrode's solution and 16 cc. of the buffer mixture. The second lot was delivered in a thin stream from a pipette into 150 cc. of boiling Tyrode's solution, which was maintained at the boiling point throughout the addition of the extract and boiled for 1 minute after all the extract had been added. The whole was then rapidly cooled in ice-cold water, transferred to a graduated cylinder, made to 400 cc. with Tyrode's solution and 16 cc. of buffer mixture were added. The solution was then mixed by shaking and filtered through glass-wool. When 5 cc. lots of the boiled and unboiled extract were incubated it was found that the rate of creatinine formation during 24 hours as determined at 2 hour intervals, was slightly greater in the former than in the latter. Moreover, the boiled extract did not show the periodic changes in rate of creatinine formation shown in the unboiled extract. The total creatinine content of the two sets of extract was the same. The creatinine was slightly increased by boiling.

However, it seemed possible that there might be produced catalyzing agents in the boiled Tyrode's solution from the well known destructive action of alkalies on glucose. Consequently a

CHART 2. Comparison of the creatinine in boiled and unboiled muscle extracts when incubated under toluene for 12 hours at 38°. Analyses were made every 2 hours. Also record of the precipitate obtained by centrifugation of 5 cc. samples of unboiled extract.

lot of 400 cc. of extract was prepared using, this time, distilled water instead of Tyrode's solution as the diluent for the macerated tissue. The procedure described in the preceding experiment was followed, save that the diluent for the unboiled extract and the extract which was boiled was 0.1 per cent Na_2CO_3. After the boiled sample had been cooled and made to the desired volume, there were added to both the boiled and the unboiled extracts the amounts of salts equivalent to those present in an equivalent volume of Tyrode's solution. After buffering, 5 cc. lots of each

TABLE IV.

Effect of Boiling on the Formation of Creatinine from Creatine in Muscle Extracts Incubated at 38°C.

	I					II				
	Creatinine.			Total creatinine.		Creatinine.			Total creatinine.	
Hours	Boiled.	Unboiled.	Difference.	Boiled.	Unboiled.	Boiled.	Unboiled.	Difference.	Boiled.	Unboiled.
	mg.	mg.	per cent	mg.	mg.	mg.	mg.	per cent	mg.	mg.
Fresh.	0.042	0.031	35.6	4.39	4.37	0.045	0.034	32.4	4.67	4.63
12	0.164	0.124	32.4	4.39	4.37	0.156	0.135	15.5	4.57	4.63
24	0.226	0.233	− 5.3	4.41	4.37	0.232	0.241	− 6.4	4.65	4.65
48	0.371	0.365	2.6	4.37	4.41					
72	0.473	0.480	− 1.4	4.35	4.41					
96	0.588	0.594	− 0.8	4.35	4.41	0.746*	0.744*	1.1	4.63	4.63
120	0.738	0.728	1.4	4.41	4.41					

* Temperature rose to 39°C.

were incubated as usual. The results of the creatinine determinations are plotted on Chart 2. There were no changes in total creatinine.

It is quite evident that the removal of the colloids has done away with the abrupt changes in the rate of creatinine formation. It is also evident that the rate of creatinine formation in the boiled extract runs along with that of the unboiled extract and at times is slightly greater. The course of the reaction in both extracts is practically the same for days thereafter as shown in Table IV.

This series of experiments gave rise to the idea that possibly the cause of the abrupt changes in rate of creatinine formation in the muscle extract might be an adsorption of the creatinine by the colloids.

Therefore, a large amount of extract was prepared. 15 cc. lots were put into a series of test-tubes and 5 cc. lots into another. After incubation for 4 or 6 hours the 15 cc. lots were centrifuged and creatinine and total creatinine determined in 5 cc. lots of the supernatant liquid, the 5 cc. of precipitated material, and the uncentrifuged extracts. The results are given in Table V.

TABLE V.

Absorption of Creatinine by the Colloids Precipitated on Centrifugation of the Muscle Extracts.

Series.	Fresh extract.		Centrifugation after 4 hours incubation at 38°C.							
			Creatinine.					Total creatinine.		
	Creati-nine	Total creati-nine.	A In pre-cipi-tate.	B In super-natant fluid	Differ-ence be-tween A and B.	C Not centri-fuged	Differ-ence be-tween A and C.	In pre-cipi-tate.	In super-natant fluid.	Not centri-fuged.
			mg.	mg.	per cent	mg.	per cent	mg.	mg.	mg
I	0.036		0.087	0.081	7.4					
II	0.038	4.23	0.693	0.080	16.2	0.081	14.8	4.25	4.27	4.30
III	0.039	4.04	0.105	0.085	23.5	0.083	26.5	4.00	4.02	4.02
Centrifugation after 6 hours incubation at 38°C.										
IV	0.038	4.23	0.122	0.105	16.1	0.105	16.1	4.28	4.30	4.27
V	0.039	4.04	0.156	0.131	19.1	0.125	24.8	4.04	4.05	4.04

It is seen that there is definite adsorption of creatinine but not of creatine. The absolute amount adsorbed is too small to be detected in the values for total creatinine since the delicacy of this method is not of the same order as is that for creatinine.

The results of the foregoing experiments demonstrate that the periodicity in rate of creatinine formation during the first 24 hours of incubation is due to changes in the state of the colloids in the muscle extracts and that adsorption of creatinine may play a significant rôle. They are suggestive in their general physiological relationships. For not only is it true that changes in chemical composition of a colloid-containing solution influence

the state of the colloids therein, but it is also here demonstrated that changes in the state of colloids influence the rate of chemical reaction.

The creatinine formation during a period of 264 hours autolysis was studied. The contents of the tubes were kept constant at 5 cc. by the addition of a few drops of distilled water when necessary. Daily additions of 0.5 cc. of toluene were made. No changes in total creatinine occurred. Thus there is no evidence of there being present in muscle extract any enzyme which de-

TABLE VI.

Velocity Constants ($k = \frac{1}{t} \log e \frac{a}{a-x}$) of the Formation of Creatinine from Creatine in Muscle Extracts during Incubation under Toluene at 38°C.

	A			B	
Creatine, mg....	$a = 5.48$			$a = 6.78$	
Time (t).	Creatinine (x).	Constant (k).	Time (t).	Creatinine (x).	Constant (k).
hrs.	mg.		hrs.	mg.	
34	0.218	0.00163	24	0.264	0.00163
48	0.331	0.00129	48	0.418	0.00137
72	0.485	0.00138	75	0.550	0.00119
100	0.649	0.00137	96	0.643	0.00110
120	0.743	0.00133	120	0.746	0.00106
144	0.808	0.00123	144	0.831	0.00099
168	0.920	0.00122	168	0.944	0.00098
192	1.000	0.00119	192	1.020	0.00094
216	1.090	0.00116	216	1.110	0.00093
240	1.180	0.00115	243	1.230	0.00093
264	1.300	0.00117	264	1.338	0.00095

stroys or forms creatine or destroys creatinine. The velocity constants of the reaction, creatine-creatinine, are given in Table VI, together with the data from which they were calculated by the formula for monomolecular reactions. While k is originally high it decreases to an approximately uniform value. It is plain that the reaction, in its later stages at least, is monomolecular. It is my belief that the changes in the value of k are due to the disturbing influence of the colloids of the extract.

The writer, therefore, concludes that *there are no enzymes present in or produced by muscle tissue as such, which form or destroy*

creatine or creatinine or affect the transformation of creatine to creatinine.

The formation of creatinine from creatine is, however, a catalyzed reaction. It is a type of catalysis that I will call "biocatalysis." This differentiation is justified by the fact that it is neither enzymatic catalysis, nor catalysis as usually understood, in that the active agent is not an added foreign substance but is the milieu of the living tissue.

Biocatalysis is the non-enzymatic catalysis of a reaction by the milieu afforded by living tissue, in which the soluble organic and inorganic constituents and the state of the colloid material play the significant rôle. It is possible that some of what has been hitherto designated as enzyme activity may be found on closer analysis to be biocatalysis.

With regard to the origin of the urinary creatinine it is quite clear that although no enzyme activity is concerned, muscle tissue may afford a milieu in which the transformation of creatine to creatinine occurs by virtue of the biocatalytic properties of its constituents. Hence the probability that this change occurs in the organism and gives rise to the urinary creatinine is further supported.

CONCLUSIONS.

1. Creatinine and creatine are easily dialyzable substances.

2. The transformation of creatine to creatinine in muscle extracts is a reaction of the first order which is masked in its early stages by changes taking place in the state of the colloids of the extracts.

3. From the fact that the amount of creatinine formed in dialyzed extracts is but slightly greater than that formed in the dialysates of such extracts, and from the fact that the increased creatinine formation in non-centrifuged extracts as compared with centrifuged extracts on incubation is also small, and from the fact that the rate of creatinine formation in boiled extracts is no less than that of unboiled extracts it is concluded that no enzyme participates in the transformation of creatine to creatinine in muscles.

4. As no changes occur in the total creatinine content of muscle extracts in any of the procedures described it is concluded that

there is no enzyme in muscle tissue which destroys or forms creatine or creatinine.

5. From the fact that the rate of transformation of creatine to creatinine in muscle extract is some three or four times as great as that taking place in water solutions of the pure compound, and from the results of the experiments detailed in this paper, it is clear that muscle tissue provides a milieu particularly favorable for this change. It is suggested that such types of acceleration be designated as biocatalytic in distinction from those brought about by enzymes and those induced by the addition of substance foreign to the organism.

BIBLIOGRAPHY.

1. Gottlieb, R., and Stangassinger, R., *Z. physiol. Chem.*, 1907, lii, 1.
2. Voegtlin, C., and Towles, C., *J. Biol. Chem.*, 1911, ix. p. xi.
3. Myers, V. C., and Fine, M. S., *J. Biol. Chem.*, 1915, xxi, 583.
4. Hammett, F. S., *J. Biol. Chem.*, 1921, xlviii, 127.
5. Hammett, F. S., *J. Biol. Chem.*, 1921, xlviii, 133.
6. Loeb, J., *J. Gen. Physiol.*, 1921–22, iv, 213, 617.
7. Bredig, G., and Weinmayr, J., *Z. physik. Chem.*, 1903, xlii, 601.
8. Bray, W. C., *J. Am. Chem. Soc.*, 1921, xliii, 1262.
9. du Noüy, P. L., *J. Exp. Med.*, 1922, xxxv, 575.
10. Davis, C. E., Oakes, E. T., and Browne, H. H., *J. Am. Chem. Soc.*, 1921, xliii, 1526.

AN ANALYSIS OF CAMEL'S COLOSTRUM.

By HELEN L. FALES.

(From the Laboratories of The Rockefeller Institute for Medical Research and the Babies' Hospital, New York.)

Through the courtesy of Doctor W. T. Hornaday of the New York Zoological Society we were able to obtain for analysis a sample of the colostrum of a Bactrian camel, 2 days after parturition. Since reports of analyses of milks, other than those of the cow and goat, are none too plentiful it seems of possible value to have a published record of this analysis.

Colostrum from Bactrian Camel—2nd Day after Parturition.

Volume of sample...165 cc.
Appearance ....Thick and rich, not yellow but creamy white.
Taste ....Bland, less taste than cow's milk. Absolutely no
    unpleasant odor.
Reaction ......Slightly amphoteric to litmus, acid reaction more
    marked.
Specific gravity..1.038
Fat..7.4 per cent
Sugar..4.2   "   "
Protein..5.4   "   "
    Casein..4.1   "   "
    Albumin...0.5   "   "
    Globulins, etc......................................0.8   "   "
Ash..0.893
CaO..0.272
MgO..0.025
P_2O_5..0.318
K_2O...0.164
Na_2O..0.082
Cl...0.128

EFFECTS OF ETHER ANESTHESIA ALONE OR PRECEDED BY MORPHINE UPON THE ALKALI METABOLISM OF THE DOG.*

By R. L. STEHLE, W. BOURNE, and H. G. BARBOUR.

(*From the Department of Pharmacology, McGill University, Montreal, Canada.*)

The purpose of the experiments to be described was to obtain further light upon the interrelations between the alkali reserve and hydrogen ion concentration of the blood and the sodium and potassium content of the urine during the anesthesia produced by ether alone, as well as by ether and morphine together. Concerning the alkali reserve of the blood during ether anesthesia, in view of data already available, no doubt exists that the bicarbonate becomes lowered in this condition. Furthermore, evidence is accumulating (1) that ether anesthesia increases the hydrogen ion concentration of the blood.

The rate of sodium and potassium excretion by the kidneys in the normal and anesthetized animal is interesting in connection with some of the explanations which have been given to account for the low alkali reserve of anesthesia. If the diminution is due to the neutralization of the bicarbonate by organic acids resulting from incomplete oxidation, some increase in the sodium and potassium content of the urine might be expected. If, on the other hand, the alkali during anesthesia merely leaves the blood for the tissues in a compensatory fashion, to maintain a constant CO_2: $NaHCO_3$ ratio, then one would expect no change in the rate of alkali excretion.

Methods.

Dogs weighing 10 to 15 kilos were employed. In some cases they were maintained upon a uniform diet consisting of ground

beef heart, crackers, and agar-agar; in other experiments the
animals were kept fasting. After two 24 hour control periods
blood was drawn and the animal was then anesthetized by placing
it in a glass box which could be closed tightly. Air saturated
with ether vapor was blown through the box. In this manner
the animal succumbed so easily (in 4 to 6 minutes) that there was
practically no struggling. As soon as anesthesia was complete
the animal was placed upon an operating table, and by intra-
pharyngeal administration of ether kept in the first stratum of
the third stage of anesthesia. Blood was again drawn immediately
and later at the times mentioned. The minimum period of ether
administration was 3 hours. The modifications of the procedure
in the morphine-ether experiments may readily be understood
from the tables.

Blood CO_2 capacity was determined by the method of Van
Slyke; hydrogen ion concentration colorimetrically by the method
of Dale and Evans (2).

Sodium and potassium in the urine were weighed as the combined sul-
fates and potassium was then determined in the mixture by the cobaltic
nitrite method described by Drushel (3). To obtain the mixed sulfates
the urine was ashed by adding it dropwise to boiling sulfuric acid in a
Pyrex Kjeldahl flask. The sulfuric acid was then transferred to a 6 inch
evaporating dish, and after evaporating the water on a water bath the
excess sulfuric acid was driven off with a free flame. The residue was taken
up with water and saturated barium hydroxide solution was added until
there was no further precipitation. The volume was then made up to
100 cc., centrifuged, and a suitable portion taken for continuing the anal-
ysis. To this portion sulfuric acid was added to remove the barium, and
after concentrating it on a hot-plate the barium sulfate was filtered off with
a Gooch crucible. The filtrate was concentrated, evaporated in platinum,
and ignited.

Ether Experiments.

The results of anesthesia with ether alone are presented in
Tables I and II (fed dogs) and III and IV (fasted dogs).

H ion Concentration of the Blood.—In Experiments 1, 6, and 8
the hydrogen ion concentration was increased after 2 hours of
ether anesthesia; in Experiment 2 no decided increase occurred
but there was a tendency in that direction. The changes noted
are considerably smaller than those reported by Atkinson and
Ets (1). It is worth noting that the induction of the anesthesia
itself produced no change in reaction.

TABLE I.

Experiment 1.

Period.	Urine.*				Blood.			
	Condition.	$Na_2SO_4 + K_2SO_4$	Na_2SO_4	K_2SO_4	Jan. 18		pH	CO_2
1	Normal.	3 65	2 05	1.60	*a.m.* 11 45	Blood.	7 50	59
2	"	3 41	1 85	1.56	*p.m.* 12 14	Ether begun.		
3a	Ether.	0.138	0 068	0 07	12 30	Blood.	7 50	45
3a	Calculated to 24 hr. basis.	0 662	0 326	0.336	2 35	"	7.30	41
3b	Part of urine lost.				3.25	"	7.37	
3a + 3b					3.50	" Ether discontinued.		32
4	Normal.	3 24	1.67	1.57				

* Animal catheterized at 11.00 a.m., daily.

TABLE II.

Experiment 2.

Period.	Urine.*				Blood.			
	Condition.	$Na_2SO_4 + K_2SO_4$	Na_2SO_4	K_2SO_4	Jan. 25		pH	CO_2
1	Normal.	3 12	1 86	1.26	*a.m.* 11 35	Blood.	7.38	47
2	"	2.93	1 45	1 48	*p.m.* 12.14	Ether begun.		
3a	Ether.	0.392	0.210	0.182	12.34	Blood.	7.44	42
3a	Calculated to 24 hr. basis.	2.00	1.06	0.920	2 36	"	7 35	
3b	Remainder of day.	3.23	1.66	1.57	2.41	"		32
3a + 3b	Total anesthesia day.	3 62	1 87	1 75	3 35	" Ether discontinued.	7 32	29
4	Normal.	2.93	1 51	1 42				

* Animal catheterized at 11.00 a.m., daily.

TABLE III.

Experiment 6.

Period.	Condition.	Na₂SO₄ +K₂SO₄	Na₂SO₄	K₂SO₄	Apr. 1		pH	CO₂
					Urine.*		Blood.	

Period.	Condition.	$Na_2SO_4 + K_2SO_4$	Na_2SO_4	K_2SO_4	Apr. 1		pH	CO_2
1	Normal.	1.50	0 12	1.38	*a.m.* 11.00	Blood.	7.50	47
2	"	0.90	0.07	0.83	11.55	Ether begun.		
3a	Ether.	0 078	0.041	0 037	*p.m.* 12.15	Blood.	7.57	36
3a	Calculated to 24 hr. basis.	0.546	0.283	0.255	2 00	"	7.34	22
3b	Remainder of day.	1.53	0 45	1 08	3.05	" Ether discontinued.	7.28	19
3a + 3b	Total anesthesia day.	1.608	0 491	1 117				
4		0.568	0 00	0 568				

* Animal catheterized at 11.30 a.m., daily.

TABLE IV.

Experiment 8.

Period	Condition.	$Na_2SO_4 + K_2SO_4$	Na_2SO_4	K_2SO_4	May 19		pH	CO_2
			Urine.*			Blood.		

Period	Condition.	$Na_2SO_4 + K_2SO_4$	Na_2SO_4	K_2SO_4	May 19		pH	CO_2
1	Normal.	1.14	0.545	0.595	*a.m.* 11.40	Blood.	7.42	
2	"	1.50	0.646	0.854	*p.m* 12 23	Ether begun.		
3a	Ether.	0.032	0.000	0.032	12 45	Blood.	7.37	30
3a	Calculated to 24 hr. basis.	0.154	0.000	0.154	2 25	"	7.29	24
3b	Remainder of day.	1.90	0.51	1.39	3.30	" Ether discontinued.	7.26	17
3a + 3b	Total anesthesia day.	2.05	0.51	1.54				
4	Normal.	0.598	0.242	0.356				

* Animal catheterized at 11.30 a.m., daily.

Alkali Reserve of the Blood.—This was lowered in all four ether experiments. The decline appears to begin soon after the induction of anesthesia and in advance of the increase in hydrogen ion concentration. The CO_2 decrease occurred earlier than in Carter's (4) experiments.

Sodium and Potassium of the Urine.—During ether anesthesia the rate of sodium and potassium excretion was greatly decreased. The most plausible explanation for this appears to be the anuria which accompanies ether anesthesia (5).

In the postanesthetic period the rate of sodium and potassium excretion was, however, increased, and to such an extent that the total excretion of these alkalies for the experimental day was abnormally high. The latter fact is most apparent in the fasting experiments (Experiments 6 and 8). In these the alkali loss suffered is reflected in the low figures for the 4th day (normal). This may be due to the greater physiological necessity for conserving these two ions than exists in the experiments in which the animals were fed a diet providing an abundance of sodium and potassium. Experiment 6 was performed upon a pregnant animal, near term, and Experiment 8 upon the same animal about 3 weeks after delivery. The tenacity with which sodium is conserved is well shown, in the first instance especially.

A possible explanation for the postanesthetic increase in sodium and potassium excretion is that during the period of anesthesia acid substances are formed and retained in the body as salts until the discontinuance of the ether. The present experiments afford no light on the nature of these acids, or even upon their existence.

Morphine-Ether Experiments.

The results of anesthesia with ether preceded by morphine are presented in Tables V and VI (fed dogs) and VII and VIII (fasted dogs).

Alkali Reserve of the Blood.—Whereas when morphine is administered alone the alkali reserve actually increases (6), this result of our experiments was influenced by the concomitant and opposite action of ether, so that practically no change occurred. Experiment 7 was an exception, a decided diminution in the alkali reserve being noted.

TABLE V.
Experiment 3.

Period.	Condition.	Na₂SO₄ + K₂SO₄	Na₂SO₄	K₂SO₄	Feb. 2		pH	CO₂
					a.m.			
1	Normal.	3 04	1.06	1.98	10 30	Blood.	7.50	59
2	"	2 21	1.36	0 85	11 10	Morphine.		
3a	Ether.	1 80	1 40	0 396	11.55	Blood.	7.40	54
					p.m.			
3a	Calculated to 24 hr. basis.	9 54	7.42	2 10	12 20	Ether begun.		
3b	Remainder of day.	3.07	1 04	2.03	12.35	Blood.	7.40	57
3a + 3b	Total anesthesia day.	4.87	2.44	2.426	2.20	"	7.35	59
					3 30	"	7.45	
					3.42	" Ether discontinued.		58

Note: columns grouped under "Urine.*" (Condition; Na₂SO₄ + K₂SO₄; Na₂SO₄; K₂SO₄) and "Blood." (Feb. 2; ...; pH; CO₂)

* Animal catheterized at 11.30 a.m., daily.

TABLE VI.
Experiment 4.

Period	Condition.	Na₂SO₄ + K₂SO₄	Na₂SO₄	K₂SO₄	Mar.17		pH	CO₂
					a.m.			
1	Normal.	3 53	1.90	1 63	10 00	Blood.	7.50	50
2	"	3 62	2.30	1.32	10.55	Morphine.		
					p.m.			
3a	Ether begun.	1 29	0 76	0 53	12.10	Blood.	7.34	53
3a	Calculated to 24 hr. basis.	7 74	4 56	3.18	12 12	Ether begun.		
					12.30	Blood.	7.23	61
					1 50	"	7.33	
					2.30	"	7.20	47
					3.30	" Ether discontinued.	7.25	50

Note: columns grouped under "Urine.*" (Condition; Na₂SO₄ + K₂SO₄; Na₂SO₄; K₂SO₄) and "Blood." (Mar.17; ...; pH; CO₂)

* Animal catheterized at 11.30 a. m., daily.

TABLE VII

Experiment 5.

Period.	Urine.*				Blood.			
	Condition.	$Na_2SO_4+K_2SO_4$	Na_2SO_4	K_2SO_4	Mar 23		pH	CO_2
					a.m.			
1	Normal.	0 83	0 22	0 61	10 00			47
2	"	0 83	0 14	0 69	10 55	Morphine.		
					p.m.			
3a	Ether.	1 10	0 38	0 72	12 15	Blood.		41
3a	Calculated to 24 hr. basis.	6 60	2 28	4 32	12 18	Ether begun.		
3b	Remainder of day.	1 73	0 15	1 58	12 30	Blood.	7 32	40
3a + 3b	Total anesthesia day.	2 83	0 53	2 30	2 30	"	7 32	46
4	Normal.	0 23	0 10	0 13	3 30	" Ether discontinued.	7 34	42

* Animal catheterized at 11.30 a.m., daily.

TABLE VIII.

Experiment 7.

Period.	Urine.*				Blood			
	Condition.	$Na_2SO_4+K_2SO_4$	K_2SO_4	Na_2SO_4	K_2SO_4	Apr 6	pH	CO_2
						a.m.		
1	Normal.	0 523	0 078	0 445		10 00 Blood.	7 39	42
2	"	0 706	0 071	0 635		10 30 Morphine.		
3a	Ether.	1 22	0 398	0 822		11 50	7.37	40
3a	Calculated to 24 hr. basis.	7 32	2 388	4 932		11 53 Ether begun.		
						p m		
3b	Remainder of day.	2 10	0 30	1 80		12 10 Blood.	7 40	43
3a + 3b	Total anesthesia day.	3 32	0 698	2 622		2 15 "	7 36	28
						3 20 " Ether discontinued.	7 35	30

* Animal catheterized at 11.30 a.m., daily.

H Ion Concentration of the Blood.—The tendency for the hydrogen ion concentration to increase was less marked than when ether alone was administered; though in Experiment 4, with no diminution of the alkali reserve, there was a lowering of the pH value.

Sodium and Potassium of the Urine.—The increase in the rate of sodium and potassium excretion is striking. The explanation of this reversal of the usual effect of ether is not obvious. Conceivably, the high CO_2 content of the blood, consequent to depressed respiration might result in drawing alkali from the tissues, and if the kidney threshold for alkali was exceeded, sodium and potassium would pass into the urine. The blood alkali is not increased, however, so that this explanation is not a likely one. In this connection the protective effect of alkalies against ether anuria, described by MacNider (5), is of interest, but it is impossible to state at present whether or not the antidiuretic action of ether can be prevented by morphine.

BIBLIOGRAPHY.

1. Atkinson, H. V., and Ets, H. N., *J. Biol. Chem.*, 1922, lii, 5.
2. Dale, H. H., and Evans, C. L., *J. Physiol.*, 1920–21, liv, 167.
3. Drushel, W. A., *Am. J. Sc.*, 1908, xxvi, 555.
4. Carter, W. S., *Arch. Int. Med.*, 1920, xxvi, 319.
5. MacNider, W. deB., *J. Pharmacol. and Exp. Therap.*, 1920, xv, 249.
6. Hjort, A. M., and Taylor, F. A., *J. Pharmacol. and Exp. Therap.*, 1919, xiii, 407.

THE METABOLISM OF SULFUR.

V. CYSTEINE AS AN INTERMEDIARY PRODUCT IN THE METAB-OLISM OF CYSTINE.*

By HOWARD B. LEWIS and DANIEL A. McGINTY.

WITH THE COOPERATION OF LUCIE E. ROOT.

(*From the Laboratory of Physiological Chemistry, University of Illinois, Urbana.*)

The ease with which cysteine is oxidized to cystine has resulted in some confusion as to which of these two sulfur-containing amino-acids is present in the protein molecule and is an active constituent of tissues. Cystine is the form which is isolated from proteins on hydrolysis. However, Heffter (1) and later Arnold (2) showed that practically all tissues gave a positive reaction with sodium nitroprusside and ammonia, a test which seemed to be specific for the mercapto group, -SH. Inasmuch as he found this reaction positive with tissue extracts free from protein, Arnold (3) suggested that cysteine was the substance present in all tissues, which was responsible for the nitroprusside reaction.

In consequence of these facts and others, the belief that cysteine was an active constituent of tissues gained credence. Direct proof of its existence in the organism, however, was lacking until Hopkins (4) in 1921 isolated a dipeptide of glutaminic acid and cysteine from tissues, to which he gave the name "glutathione." He considered that this substance was important in tissue oxidation and reduction and that the mechanism of its reaction consisted in a shifting of the equilibrium between cysteine and cystine according to the activities of the cell. He believed it probable that the significance of the occurrence of cysteine as a constituent of a peptide lay in the fact that it was thereby protected from catabolic changes.

Another line of evidence which indicates that cysteine is an active substance in metabolism is also available. Jaffe (5) and Baumann and Preusse (6) as well as others have shown that when monohalogen derivatives of benzene, notable bromobenzene, are administered to a dog, there is excreted bromophenyl-mercapturic acid, a derivative of cysteine. However, in the absence of protein in the diet, this synthesis does not take place (7). Further work (8) showed that if cystine be injected subcutaneously into dogs on a protein-free diet, administration of bromobenzene gives rise to mercapturic acid. These results were interpreted to mean that in animals whose diets were deficient in protein content, the cystine of endogenous origin does not pass through the same path in catabolism.

It must be borne in mind, however, in considering these results, that the cysteine is produced as the result of a demand for a detoxicating substance and that its formation may be due to a deviation from the normal path of catabolism in response to abnormal conditions; *i.e.*, the introduction of a toxic benzene derivative into the system. A similar question is raised as to the glycuronic acid used for the detoxication of many foreign organic compounds. It is still debatable whether glycuronic acid, if formed from glucose, is a normal decomposition product or a substance originating in a specialized metabolic process for detoxication.

The purpose of the present communication is to report the occurrence of a cysteine derivative in the urine after the administration of a non-toxic derivative of cystine in which complete oxidation of the molecule was prevented by "protecting" the amino group by substitution. The results support the work of other investigators and indicate that cysteine is a normal stage in the catabolism of cystine in the animal body.

In a previous paper (9), it was shown that if the amino group of cystine was protected from deamination by conjugation with phenylisocyanate, the resulting phenyluraminocystine was not oxidized normally and the greater part of the sulfur of the complex was recovered in the urine as unoxidized sulfur. Cystine under similar conditions was completely oxidized and the sulfur appeared in the urine as sulfates.

During the analyses of the urines for total sulfur, it was observed that, on the days of administration of the phenyluramino-

cystine, the addition of the copper reagent of Benedict to the urine resulted in the formation of a grey-black precipitate. Although the color was slightly different, the substance was considered to be copper sulfide. No such phenomena were observed in normal urines. After the completion of the analyses, we returned to the study of this precipitate and found that after a period of 2 weeks to a month, the precipitate with the copper reagent was obtained in small amounts or was completely lacking. This suggested that we might be concerned with a derivative of cysteine which had been oxidized to cystine on standing. A systematic study of the substance was then made.

The phenyluraminocystine, usually in amounts of 1.0 gm., was administered to rabbits either *per os* or subcutaneously in water suspension or as the sodium salt. The urine was collected for 24 hours after the administration of the phenyluraminocystine.

It was observed that the experimental urines gave an intense purple color with sodium nitroprusside and ammonia (1, 2), a reaction not shown by the normal urines, and that it was possible to remove all the material which gave this reaction by extraction with ether.[1] The urine was acidified and filtered or centrifuged to remove any precipitate (of unchanged phenyluraminocystine?) which might be formed. This precipitate did not react with nitroprusside and ammonia. The liquid was then extracted three times with ether (2 volumes of ether to 1 volume of urine). Occasionally some difficulty was experienced because of emulsions, but when these occurred, they were readily broken by centrifugation. The extracted urine gave no reaction with nitroprusside and ammonia although the test for creatinine in which sodium

[1] Quantitative evidence as to the removal of the organic sulfur compound by ether was obtained by analysis of the urine of a rabbit following administration of phenyluraminocystine. Before extraction with ether, the urine contained 0.146 gm. of total sulfur, and 0.088 gm. of unoxidized sulfur (by difference); after extraction, the total sulfur was reduced to 0.085 gm. and the unoxidized sulfur to 0.027 gm. Before extraction the unoxidized sulfur comprised slightly over 60 per cent of the total sulfur, while after treatment with ether only 31 per cent of the total sulfur was present as unoxidized sulfur, a figure which is only slightly higher than the normal for a rabbit on the type of diet fed. This demonstrates that by far the greater part of the "extra" unoxidized sulfur present was in the form of an ether-soluble compound.

hydroxide is substituted for ammonia was still positive. Creatinine does not react with sodium nitroprusside and ammonia.

After the evaporation of the combined ether extracts at low heat, an oily residue remained which did not crystallize readily. This oily material gave a strong nitroprusside test, a fleeting blue color with dilute ferric chloride, and a grey-black precipitate with copper sulfate. The oil dissolved readily in hot water, and on cooling formed an emulsion from which a gummy light brown mass gradually separated on standing in the ice box. When this solution was allowed to evaporate spontaneously in air, after some time, white crystals separated, and neither crystals nor solution gave a positive nitroprusside reaction. These crystals, although obviously impure, were probably phenyluraminocystine

TABLE I.

Experiment No.	Melting point.	Sulfur.
	°C.	per cent
6	162	12.8
63	159	13.9
64	159	12.8
7	159	10.4
72	158	12.7
62	163	
5	155	
Phenyluraminocystine.	159	13.38

since the melting points were similar. Melting points and analyses for sulfur of several of these impure preparations are given in Table I. It seemed probable that we were dealing with phenyluraminocysteine which had slowly been converted into phenyluraminocystine on standing in air.

Since direct isolation and purification of the unknown substance were unsuccessful, a study was made of the insoluble copper salt. This could be obtained as a grey-black precipitate from the neutral or faintly alkaline urine by cautious addition of a 3 per cent solution of copper sulfate. However, an account of the probability of inclusion of other substances in the precipitate, direct precipitation of the copper salt from the urine was considered inadvisable. Accordingly, the urine was extracted with ether as previously described and the ether extract was shaken

with a dilute (1 per cent) solution of copper sulfate.[2]   The grey-black precipitate of the copper salt which separated in the aqueous layer was drawn off from the ether in a separatory funnel, filtered, carefully washed with cold water, alcohol, and ether, and dried at 80°. Some difficulty was experienced in removing the last traces of copper salts from the precipitate.   The light grey powder obtained decomposed on heating at 170–171° (uncorrected) and left a small amount of a black residue in the melting point tube.

For the analysis of the copper salt the material was carefully ashed in a platinum crucible, the residue dissolved in nitric acid, and the copper determined iodometrically in the usual manner. Sulfur was determined either by oxidation with Benedict's reagent, as used in urine analysis or by oxidation in a Parr bomb.   The results of analysis are given in Table II.

Four copper salts of phenyluraminocysteine are possible.

(I)

$CH_2 - S$
|
|
| Cu
|
$CH - CO - O$
|
NH
|
CO
|
N$\diagdown$H
  $\diagdown C_6H_5$

(II)

$CH_2 - SH$
|
$CH - CO - O - Cu - O - CO - CH$
| |
NH   NH
| |
CO   CO
| H | H
N$\diagdown$ N$\diagdown$
  $\diagdown C_6H_5$ $\diagdown C_6H_5$

$CH_2 - SH$

(III)

$CH_2 - S - Cu - S - CH_2$
|
|
|
$CH - CO - O - Cu - O - CO - CH$
| |
NH   NH
| |
CO   CO
| H | H
N$\diagdown$ N$\diagdown$
  $\diagdown C_6H_5$ $\diagdown C_6H_5$

(IV)

$CH_2 - S - Cu - S - CH_2$
| |
$CH - COOH$ $CH - COOH$
| |
NH   NH
| |
CO   CO
| H | H
N$\diagdown$ N$\diagdown$
  $\diagdown C_6H_5$ $\diagdown C_6H_5$

---

[2] Other salts of copper, notably the acetate, were also used for the precipitation of the copper complex, but the character and composition of the washed precipitate did not differ from those obtained with copper sulfate.

Of these Formulas I and III and Formulas II and IV have the same percentage composition. Formula III is considered improbable on account of the size of the ring which would be formed. As will be seen from the table it should be easy to distinguish between the two types of salts by the differences in the percentage of copper and in the ratio of copper to sulfur. The difference in the percentage of sulfur is not great. The figures obtained on analysis indicate that the copper salt probably has the structure assigned by Formula I. While the analytical figures are not theoretical, they may be considered satisfactory in view of the difficulty in washing the bulky precipitate.

TABLE II
Analysis of the Copper Salt of Phenyluraminocysteine.

	Found.		Theory for	
			$C_{10}H_{10}O_2N_2SCu$	$C_{20}H_{22}O_6N_4S_2Cu$
	per cent		per cent	per cent
Cu	20.49 20.40 } 20.45		21.09	11.64
S	9.74 9.66 } 9.70		10.61	11.82
Cu: S	2.108		1.988	1.015

Evidence as to the nature of the derivative present in the urine after the administration of phenyluraminocystine was obtained in another way. The urine was treated with dilute copper sulfate directly and the precipitated copper salt filtered off and washed. The salt was suspended in water, the copper removed by hydrogen sulfide, and the copper-free filtrate concentrated *in vacuo.* The liquid remaining in the flask was extracted with ether and the ether evaporated at low temperature. An oily liquid which gave a strong nitroprusside reaction remained. This was dissolved in water, allowed to stand at room temperature for 24 hours, hydrochloric acid added to give a concentration of 10 per cent, and the solution boiled vigorously for 1 hour. If the oily residue contained phenyluraminocysteine, this treatment should have converted it to the hydantoin of phenyluraminocystine. The solution was cooled and allowed to stand in the air. Beautiful white needle crystals, resembling those of tyrosine although somewhat larger, separated. Neither crystals nor mother liquor

gave a positive nitroprusside test. The product was twice re-
crystallized from 50 per cent alcohol. Positive tests for nitrogen
and sulfur were obtained with the crystals. The crystals melted
at 117° (uncorrected), and when mixed with a pure sample of the
hydantoin of phenyluraminocystine which melted at 116.5–117°,
gave a mixed melting point of 115.5–116°. Inasmuch as phenyl-
uraminocystine itself is not precipitated by copper sulfate, this
experiment, which has been repeated with results similar to those
reported above, furnishes additional evidence that phenyluramino-
cystine is first broken down to phenyluraminocysteine in the
organism of the rabbit and that this latter substance is then ex-
creted in the urine, since further oxidation is prevented by con-
jugation of the amino group.

4 gm. of phenyluraminocystine were fed to a dog of 12 kilos
weight. No increase in the total or unoxidized sulfur of the urine
was observed either on the experimental or succeeding days, nor
was there present in the urine any substance which reacted with
copper sulfate to give a black precipitate. 0.5 gm. of phenylur-
aminocystine was fed to a man and the urine secreted in the next
12 hours examined. After extraction with ether as already de-
scribed, and evaporation of the ether extract, the residual oily
material gave a positive nitroprusside test, a black precipitate
with copper sulfate, and a blue color with dilute ferric chloride
solution. We have never failed to obtain evidence of the presence
of phenyluraminocysteine in the urine of the rabbit after adminis-
tration of phenyluraminocystine, regardless of the manner of
administration.

Attempts have been made to prepare phenyluraminocysteine
by the reduction of phenyluraminocystine with tin and hydro-
chloric acid. The insolubility of the latter and the ease with
which it is converted to the hydantoin in the presence of acid are
factors which interfere with the reduction. As yet we have not
succeeded in carrying out the reduction satisfactorily. The work
is being continued along these and related lines.

SUMMARY.

In the urine of rabbits to which phenyluraminocystine, a non-
toxic substance, has been fed, there is present a substance which
has been identified as phenyluraminocysteine. This furnishes

evidence that the first stage in the catabolism of cystine is conversion to cysteine, with subsequent deamination and oxidation of the latter.   In the case of the phenyluraminocysteine, deamination has been prevented by conjugation and the cysteine derivative is probably excreted as such in the urine.

BIBLIOGRAPHY.

1. Heffter, A., *Med. naturwiss. Arch.*, 1908, i, 81.
2. Arnold, V., *Z. physiol. Chem.*, 1910–11, lxx, 300.
3. Arnold, V., *Z. physiol. Chem.*, 1910–11, lxx, 314.
4. Hopkins, F. G., *Biochem. J.*, 1921, xv, 286.
5. Jaffe, M., *Ber. chem. Ges.*, 1879, xii, 1092.
6. Baumann, E., and Preusse, C., *Ber. chem. Ges.*, 1879, xii, 806.
7. Thomas, K., and Straczewski, H., *Arch. Physiol.*, 1919, 249.
8. Kapfhammer, J., *Z. physiol. Chem.*, 1921, cxvi, 302.
9. Lewis, H. B., and Root, L. E., *J. Biol. Chem.*, 1922, l, 303.

THE ADAPTATION OF THE PENTABROMOACETONE METHOD TO THE QUANTITATIVE DETERMINATION OF CITRIC ACID IN THE URINE.*

By WILLIAM B. McCLURE.

(From The Otho S. A. Sprague Memorial Institute Laboratory of the Children's
Memorial Hospital, Chicago.)

The determination of citric acid by the pentabromoacetone method[1] depends upon the formation of water-insoluble pentabromoacetone, when citric acid is oxidized by potassium permanganate in the presence of bromine. The quantitative results would, of course, be vitiated by any impurity of the pentabromoacetone.

When McClure and Sauer[2] employed in urine Kunz's[3] modification of the pentabromoacetone method for citric acid determination, they, at times, found a considerable residue upon treating the pentabromoacetone precipitate with acetone, whereas pure pentabromoacetone is readily dissolved in that solvent. They also found a considerable difference, in many cases, between results obtained by the pentabromoacetone method and those obtained by the Salant and Wise[4] method. These findings pointed out the desirability of further study of the pentabromoacetone method as applied to urine, to ascertain factors interfering with its accuracy.

---

* The early part of the work was done in conjunction with Dr. Hugh Macdonald.

[1] Stahre, L., Eine Citronensäurereaktion, Nord. farm. Tidskr., 1895, ii, 141.

[2] McClure, W. B., and Sauer, L. W., Comparison of pentabromoacetone method and Salant and Wise's method for citric acid determination in urine, Am. J. Physiol., 1922, in press.

[3] Kunz, R., Ueber das Vorkommen und die Bestimmung von Zitronensäure im Weine und den Nachweis der Zitronensäure in Milch, Marmeladen und Frucht-sirupen, Arch. Chem. u. Mikrosk., 1914, vii, 285.

[4] Salant, W., and Wise, L. E., The action of sodium citrate and its decomposition in the body, J. Biol. Chem., 1916–17, xxviii, 27.

357

The following experiment suggested a possible source of error: Urine was treated with sulfuric acid and an excess of bromine, shaken, and allowed to stand. This produced a precipitate, containing bromine, which was only partially soluble in alcohol, ether, or acetone. The alcohol, ether, and acetone solutions of this bromine precipitate were red-brown in color and became turbid upon addition of water.

Since, during the test for citric acid in the urine by the pentabromoacetone method, bromine is liberated and sulfuric acid has been added previously, the conditions are present for the formation of the bromine precipitate which was described in the preceding paragraph. Should this precipitate be produced, at least a part of it would be present as an impurity in the pentabromoacetone precipitate and would to that extent vitiate the results of the citric acid determination.

In order to free the pentabromoacetone method, as applied to urine, from an error which might be due to the appearance of this bromine precipitate, the following procedures were tried: (1) Separation of the pentabromoacetone from the bromine precipitate; (2) reduction of the amount of bromine precipitate formed; and (3) a combination of these two procedures.

Separation of the Pentabromoacetone from the Bromine Precipitate.

(*a*) Numerous trials failed to disclose a *solvent* which would serve to separate the bromine precipitate in its entirety from the pentabromoacetone precipitate. (*b*) Pentabromoacetone is readily volatilized by heat. The effect on bromine precipitate of a degree of heat sufficient to volatilize actively pentabromoacetone was tested out. To accomplish this, known quantities of bromine precipitate and pentabromoacetone, each in a porcelain crucible, were heated simultaneously in the electric oven at temperatures approximately 100–105°C. and the loss was determined in each case. Table I shows the results of four such experiments, each substance was charted separately. The heating was prolonged in Experiment 4 for the purpose of more rigorously testing the resistance of the bromine precipitate to this degree of heat.

It is seen from Table I that less than 18 per cent of the bromine precipitate was lost when the temperature was maintained at approximately 100–105°C., while the pentabromoacetone was

practically all driven off under similar conditions. However, in one experiment, which is not included in Table I, 10.1 mg. of bromine precipitate, without a pentabromoacetone control, were heated at approximately 100°C. for 24 hours with a loss of 2.5 mg.

TABLE I.

Results of Heating Simultaneously Bromine Precipitate and Penta-bromoacetone.

Experiment No.	Bromine precipitate used.	Heated.	Temperature (approximately).	Loss.	Percentage lost.
Results of heating bromine precipitate.					
	mg.	hrs.	°C.	mg.	per cent
1	90.5	$4\frac{1}{2}$	100	9.5	10.5
2	73.8	$5\frac{1}{2}$	105	9.1	12.3
3	86.5	$8\frac{1}{2}$	100	10.9	12.6
4	16.8	$39\frac{1}{2}$	105	2.9	17.3
Results of heating pentabromoacetone.					
1	88.6	$4\frac{1}{2}$	100	88.2	99.5
2	69.0	$5\frac{1}{2}$	105	68.9	99.9
3	70.6	$8\frac{1}{2}$	100	70.6	100.0
4	163.2	$39\frac{1}{2}$	105	162.9	99.8

TABLE II.

Results of Heating Mixtures of Bromine Precipitate and Pentabramoacetone.

Mixtures.	Used.	Heated	Temperature (approximately).	Loss.	Remaining.
	mg.	hrs.	°C.	mg.	mg.
Pentabromoacetone..................	52.0	$9\frac{1}{2}$	100	51.9	1.2
Bromine precipitate.	1.1				
Pentabromoacetone..................	50.3	24	100	51.8	8.7
Bromine precipitate.................	10.2				

or 24.8 per cent. This percentage of loss was unusually high and was encountered only in this one experiment.

Next, mixtures of small quantities of bromine precipitate and relatively large quantities of pentabromoacetone were heated. Table II shows the results of two such experiments.

These results are such as would be expected after consideration of the findings presented in Table I. In the first experiment the loss upon heating the mixtures was about equal to the quantity of pentabromoacetone employed. In the second experiment, where a larger quantity of bromine precipitate was used and longer heating was carried out, the loss was somewhat greater than the amount of pentabromoacetone. This latter finding is readily explainable on the basis of a partial volatilization of the bromine precipitate.

Also determinations of pentabromoacetone were made in duplicate, in two specimens of native urine with heating used as an additional step to the pentabromoacetone method. Table III gives the results of these determinations.

TABLE III.

Pentabromoacetone Obtained from Urine, Using Heat as an Additional Step in the Pentabromoacetone Method.

Normal urine, 50 cc.	Penta-bromoace-tone found	Residue after heating.	Heated.	Tempera-ture.
	mg.	*mg.*	*hrs.*	$°C.$
Mixed specimen from adults.	12.3 12.6	2.9 2.9	$22\frac{1}{2}$	100–105
Mixed specimen from children.	30.5 29.7	6.4 7.8	$18\frac{1}{2}$	100–106

Reduction of the Amount of Bromine Precipitate.

It was found that a preliminary treatment of the urine with charcoal[5] causes a marked diminution of the quantity of the bromine precipitate which is produced when urine is treated with sulfuric acid and an excess of bromine. This was true, whether the urine was boiled with sulfuric acid and charcoal, or whether the unheated urine was rendered alkaline by sodium hydroxide and only shaken with charcoal. This was demonstrated in the following way: From 150 cc. quantities of a specimen of urine, 45 mg. of bromine precipitate were obtained when charcoal was not used, and 3.9 mg. of bromine precipitate when the urine had been previously treated by boiling with sulfuric acid and charcoal

[5] Blutkohle, Kahlbaum, was used in all the experiments.

and filtered. A different specimen of urine yielded 31.3 mg. of bromine precipitate per 150 cc. without charcoal treatment, and only 0.1 mg. of bromine precipitate after being rendered alkaline with sodium hydroxide and shaken, unheated with charcoal.

Amberg and McClure[6] had found that to boil urine with sulfuric acid and charcoal may cause a considerable loss of citric acid—as much as 27 per cent in a solution containing the equivalent of 30 mg. of citric acid, in the form of its sodium salt, in 50 cc. of water. It was therefore evident that this method of using charcoal was unavailable for our purpose.

However, it was found in this study that the use of charcoal in an unheated alkaline solution caused little or no loss of citric acid. For example, by using 50 cc. quantities in duplicate, from an aqueous solution of sodium citrate containing the equivalent of 50 mg. of citric acid in each 50 cc., 51.3 and 51.0 mg. of citric acid,[7] respectively, were determined. This method of employing charcoal, therefore, serves the purpose quite satisfactorily.

Combination of Reduction in the Formation of Bromine Precipitate and Separation of Pentabromoacetone from the Bromine Precipitate.

In view of the results reported above, it seemed that a combination of a preliminary treatment of the urine by rendering it alkaline, and shaking with charcoal without heating, and of separation of the pentabromoacetone from impurities by volatilization might be advantageous. To test this out, the pentabromoacetone method for citric acid determination with these two steps added[8]

[6] Amberg, S., and McClure, W. B., The occurrence of citric acid in urine, *Am. J. Physiol.*, 1917, xliv, 453.

[7] Throughout this work in computing the amount of citric acid, 5 mg. were added for each 50 cc. of solution tested, because of an undetermined rest which Amberg and McClure[6] had found to exist.

[8] The pentabromoacetone method for citric acid in urine, as modified, is: Approximately 200 cc. of urine rendered alkaline to litmus with 5 per cent NaOH are filtered. To 150 cc. of filtrate 3 gm. of charcoal are added and the mixture is shaken vigorously unheated for 1 minute and filtered. Duplicate specimens of 50 cc. of the filtrate are taken and to each, 1 cc. of dilute H_2SO_4 is added. Bromine vapor is poured in. If the solution remains clear, 10 cc. of 1:1 H_2SO_4 and 3 cc. of 37.5 per cent KBr are added and the solution is heated in a water bath at 50–55°C. for 5 minutes. 20 cc. of 5

was applied to specimens of urine to which known quantities of sodium citrate had been added. Table IV gives the results of these experiments.

TABLE IV.

Results of Duplicate Determinations of Citric Acid,* in Urine by the Penta-bromoacetone Method, Using Preliminary Charcoal Treatment and Separa-tion of the Pentabromoacetone from Impurities in the Final Precipitate by Heat.

Experiment No.	Citric acid.				
	Added.	Recovered.	In native urine (same method).	Net recovered.	Recovered.
	mg.	*mg.*	*mg.*	*mg.*	*per cent*
1	10.0	21.6	10.3	Average 10.7	107.0
	10.0	21.4	11.2		
2	20.0	30.1	10.4	Average 19.6	98.0
	20.0	30.6	11.2		
3	30.0	43.3	13.8	Average 29.9	99.7
	30.0	44.3	14.0		
4	40.0	50.0	12.6	37.4	93.5
	40.0	50.7	10.7	40.0	100.0
5	40.0	53.3	13.2	Average 38.95	97.0
	40.0	51.1	13.3		
6	40.0	54.9	17.0	37.9	94.8
	40.0	56.3	17.0	39.3	98.0
7	50 0	63.3	13 8	Average 48.9	97.8
	50 0	62.4	14.1		

* Throughout the experiments 50 cc. quantities of the solutions being tested for citric acid, were used for each determination and bromine vapor was added instead of bromine water, following the suggestion of Amberg and McClure.[6]

per cent $KMnO_4$ solution are added drop by drop with vigorous shak-ing. Concentrated $FeSO_4$, acidified with H_2SO_4, is added, slowly with shaking, in amount sufficient to remove the undissolved $MnO_2 \cdot H_2O$ and excess of bromine. The precipitate is weighed in a Gooch crucible, after drying 24 hours in a desiccator. The crucible and contents are now heated over night in the electric oven at 100–105°C. (A much shorter period of heating is usually sufficient but necessitates reheating and an additional weighing to determine whether all the pentabromoacetone has been vola-tilized off.) After cooling the crucible is again weighed and the loss of weight due to heating is taken as equivalent to the quantity of penta-bromoacetone. From the pentabromoacetone, the quantity of citric acid + $1H_2O$ is calculated by multiplying by 0.464 and adding 5 mg., because of an undeterminable fraction.

CONCLUSIONS.

The pentabromoacetone method may be used for the quantitative determination of citric acid in normal urine with satisfactory results, if the unheated urine has been rendered alkaline, previously, by sodium hydroxide, and shaken with charcoal and filtered; and if the final precipitate is heated as a means of separating pentabromoacetone from impurities.

This work was undertaken at the suggestion of Dr. Samuel Amberg to whom I am indebted for much helpful advice.

AN ELECTROCHEMICAL STUDY OF THE CONDITION OF SEVERAL ELECTROLYTES IN THE BLOOD.

By B. S. NEUHAUSEN and E. K. MARSHALL, Jr.

(*From the Laboratory of Physiology, the Johns Hopkins University, Baltimore.*)

Whether the salts present in the blood have the same degree of ionization as in aqueous solution and whether the various ions are free or bound by the proteins have been two of the questions that have attracted the attention of physiologists and biological chemists. Answers to these questions have been sought principally by two methods—by ultrafiltration of serum with pressure or by compensatory dialysis.

Much of the early work, in which the filtration was performed under a pressure of a number of atmospheres, is open to the criticism voiced by Burian (1) that any labile compounds between the proteins and inorganic ions may have been decomposed by the excessive pressures.[1] Only recently has this difficulty been obviated by Cushny (2) and Richter-Quittner (3) who filtered serum free from the protein at pressures of 150 mm. and lower. Compensatory dialysis while it has a great many advantages is still open to the objection that it is really impossible to include all the other constituents but the one under consideration in exactly the same concentrations inside and outside of the dialyzing sac, which fact might well cause a disturbance of equilibria of the system. It seemed advisable, therefore, to attempt to answer the aforementioned question by a totally different method—the electrometric determination of the concentration of the ions. In this in-

[1] Bridgman (Bridgman, P. W., *J. Biol. Chem.*, 1914, xix, 511) obtained only a stiffening of egg white with a pressure of 5,000 atmospheres and complete coagulation at 7,000 atmospheres. Lower pressures for extended periods of time would also be effective, particularly if the disruption of these labile compounds is attended with an appreciable decrease in volume.

vestigation, determinations were made of the concentrations of sodium, chlorine, and calcium ions.

Apparatus and Methods.—The sodium ion concentrations were determined by means of a sodium amalgam electrode which is a reversible electrode with respect to sodium ions. In a detailed study of the reliability of the sodium amalgam electrode for determining sodium ions both when sodium salts are present alone and when admixed with salts of other cations, it was shown by Neuhausen (4) that in the range of concentrations of sodium present in the blood, the sodium amalgam electrode is reliable and that, furthermore, potassium and calcium ions in concentrations such as are present in the blood do not interfere. The sodium amalgam was prepared by the electrolysis of a sodium chloride solution. It contained about 0.2 per cent sodium by weight, and was contained in a dropping electrode modified somewhat from the design of Lewis and Kraus (5). This is represented in Fig. 1. The sodium amalgam electrode was connected to a generator of hydrogen and thus there was always an atmosphere of dry hydrogen at a pressure somewhat greater than atmospheric. The amalgam came in contact with the solution at the tip F. By opening the stop-cock C amalgam could be run out and the surface renewed at will. A platinum wire sealed in the glass at P, served as electrical contact between the amalgam contained in the electrode and the mercury in the side arm which was connected to the potentiometer. The rest of the cell arrangement was similar to that of a hydrogen electrode outfit, saturated KCl being used in the bridge and mercury and calomel in saturated potassium chloride being the second half cell. A Leeds and Northrup type K potentiometer and a certified Weston cell were used.

Before making a measurement on blood, the E.M.F. of the Na amalgam electrode against a 0.123 N NaCl solution was determined. From the difference in voltage obtained when the Na amalgam electrode was measured against the NaCl solution and against serum or defibrinated blood, the concentration of the Na ion could be calculated from the formula

$$E = 0.059 \log \frac{0.1026}{c}$$

in which E is the difference in voltages, 0.1026 is the concentration expressed in mols per liter of Na ion in 0.123 N NaCl, and c is the unknown concentration of Na ion in the solution. Since sodium bicarbonate in the concentration present in the blood has

Fig. 1.

a degree of dissociation of about 83 per cent according to Walker and Cormack (6) which is equal to that of 0.12 N sodium chloride, when c is divided by 0.83 the concentration of ionized and union-ized sodium figured as NaCl (and $NaHCO_3$) is obtained.

The chlorine concentration was determined by means of a silver-silver chloride electrode. A silver rod coated with silver chloride is put into the solution in which it is desired to determine the Cl ion concentration. This type of an electrode had been used in the determination of the solubility product of silver chloride and more recently by Brown and Hill (7) in a study of chlorine concentrations in serum. The electrode and solution constitute the positive half cell, while the rest of the cell was made up of a bridge of saturated potassium chloride, and a calomel half cell containing saturated potassium chloride, as in the case of the determination of the concentration of Na ions. The E.M.F. of this electrode in 0.123 N sodium chloride was determined. From the difference in voltage obtained when the electrode was measured against 0.123 N sodium chloride and against serum or defibrinated blood the concentration of the Cl ion can be calculated from the formula

$$E = 0.059 \log \frac{c}{0.1026}$$

in which c signifies the unknown Cl concentration and the other terms have the same significance as in the formula for the computation of the Na ion concentrations. By dividing c by 0.83 the concentration of ionized and unionized chlorine is obtained.

In determining the concentration of the Ca ion a number of difficulties were encountered. Since the calcium amalgam is very reactive, it had to be prepared in a special way as explained by Neuhausen (8) elsewhere. The amalgam of about 0.005 per cent Ca was kept in the electrode under an atmosphere of CO_2, and during the determination of an E.M.F. the amalgam was allowed to flow continuously from the electrode. Otherwise, the arrangement was the same as in the case of the sodium amalgam.

Another difficulty, however, did not prove quite as surmountable as the reactivity of calcium amalgam. When as in the case of a solution such as serum there are present other alkali cations besides the one for which the amalgam is a reversible electrode, there is a tendency for these cations to replace the particular alkali or alkaline earth in the amalgam, so that a mixed electrode is obtained, which is of no use in determining concentrations. The effect is especially marked in the case of serum since the concentration of the sodium is about 100 times as great as that of the calcium.

To obviate this difficulty, solutions were prepared which contained 0.123 N NaCl, 0.03 N NaHCO$_3$, 0.0025 M CaCl, and 0.0015 M MgCl. Such a solution approximates as far as disturbing effects are concerned the composition of dog's blood, the effect of potassium being included in the sodium. The E.M.F. was determined when the Ca amalgam was dropping into this solution. The E. M. F. was then determined when the amalgam was dropping into serum. From the difference in voltage obtained in the two cases the concentration of the Ca ion can be calculated from the formula

$$E = 0.0295 \log \frac{0.0025}{c}$$

in which c is the unknown concentration of the calcium ion, and the other terms have the same significance as in the formula used above.

It should be noted that the calculation of the calcium concentration is valid only if it is certain that the calcium electrode in the solution approximated in every way the action of the calcium amalgam in serum. This cannot be claimed absolutely, so that an error as large as 25 per cent may be inherent in this method. Other methods are being worked out which may do away with the use of a calcium amalgam electrode for determining the calcium ion.

The sample of blood or serum was obtained from dogs in some cases (6, 7, 8) without the use of anethesia and in others with the aid of paraldehyde or ether. No difference was noticed in the ionic concentration in either case.

Results.—The results obtained in this investigation on serum are given in Table I, under headings which are self-explanatory. The quantitative analysis of the sodium, calcium, and chlorine were made by the Department of Pediatrics of this University through the courtesy of Professor Howland and Doctor Kramer, whose kindness is hereby acknowledged.

On examination of Table I it is apparent that all the sodium and chlorine found in the blood can be accounted for within experimental error on the assumption that the sodium and chlorine in the serum are present as sodium chloride and sodium bicarbonate. This fact is in agreement with the finding of Neuhausen (9) that on the assumption of a similar degree of dissociation for salts in the serum as for aqueous solutions of equal concentration the

observed and calculated lowering in vapor pressure and lowering in freezing point of the serum are in good agreement. These observations in themselves do not rule out the possibility of the presence of Na-protein compounds in the blood, for it could be maintained that the Na-proteinates have the same degree of ionization as the bicarbonate. In fact Rona and György (10) claimed to have confirmed by dialysis Hamburger's (11) contention that about 15 per cent of the total sodium was in an indiffusible form. Against the conclusion of these authors may be cited the results obtained by Cushny who by filtering ox serum free from colloids at a pressure of only 150 mm. of mercury found that all the non-colloid constituents of the serum except Ca and Mg are in simple

TABLE 1.

Sample No.	Difference in E. M. F. of Ag-AgCl electrode in sample and 0.123 N NaCl.	Total chlorine normality calculated.	Total Cl normality found by analysis.	Difference in E. M. F. of Na electrode in sample and 0.123 N NaCl.	Total Na normality calculated.	Total Na normality found by analysis	Difference in E. M. F. of Ca electrode in sample and 0.002 M CaCl$_2$	Total Ca molarity calculated.	Total Ca molarity found by analysis.
1	−0.0027	0.1112	0.1117	−0.0066	0.1609	0.1495			
2	−0.0029	0.1103	0.1127	−0.0070	0.1624	0.1586			
3	−0.002	0.1142	0.1140	−0.1169	0.1617	0.1582			
4	−0.0029	0.1103	0.1117	−0.0070	0.1624	0.1586			
5	−0.002	0.1142	0.1145	−0.0065	0.1592	0.1494	+0.020	0.000524	0.0025
6	−0.003	0.1100	0.1071	−0.0060	0.1562	0.1556			
7	−0.0029	0.1103	0.1093	−0.0055	0.1533	0.1491	+0.0193	0.000554	0.00245
8	+0.001*	0.1227	0.1081	−0.0060	0.1562	0.1521	+0.019	0.000567	0.00252

* Some potassium chloride from the bridge flowed over.

solution in the blood. Richter-Quittner (3) found that all the sodium could be filtered from serum at low pressures. These facts indicate that it is unlikely that there is any appreciable quantity of bound sodium in the serum. Evans (12) has shown by a careful calculation that at a pressure of 40 mm. CO_2 there is no margin left from the total cations present to combine with the proteins. In this work it was found that the concentration of sodium ions does not change more than 1 to 2 per cent when the pressure of CO_2 is varied from 0 to 40 mm. The sodium apparently is not bound to any appreciable extent.

Most investigators agree at present that only a small portion, if any, of the chlorine in the serum is in a bound or non-diffusible

form. This binding observed in diffusion is dependent on the pH of the solution and can be accounted for on the basis of a Donnan membrane equilibrium existing on the two sides of the filter. Because of the greater quantity of chlorine found in plasma when the ashing method is used than when the precipitation of the proteins is used, Falta and Richter-Quittner (13) postulated the existence of a compound of fibrinogen and chlorine. No such difference appears in the use of the two methods in the analysis of serum. These observed differences in plasma have been partly explained, on the difference of colloidal condition, so that these authors have recently declared (14) that the existence of the fibrinogen-chloride compound is an open question. Van Creveld (15), however, has proved by analysis of the aqueous humour, which may be considered as a dialysate of blood, that there is actually more total chlorine in the aqueous humour obtained on the first puncture which is practically protein-free than in the liquid of the second puncture which contains 2 to 5 per cent protein. Van Creveld concludes, therefore, that no appreciable quantity of chlorine is bound. Rona (16) by compensatory dialysis and Asher and Rosenfeld (17) by diffusion of serum against water concluded that all of the chlorine is present in a diffusible form. Cushny and Richter-Quittner have both found, moreover, that all of the chlorine can be completely filtered at low pressures. The results in Table I bear out the contention of those who have claimed that no appreciable quantity of the chlorine is bound. It was found, moreover, that the pressure of CO_2 had no influence on the chlorine concentration in serum. In defibrinated blood slight variations were observed with varying CO_2 pressure. As both serum and whole blood can be used with the sodium amalgam and silver chloride electrodes, these methods are feasible for clinical investigations.[2]

From the results in Table I it appears that about 80 ± 5 per cent of the calcium is present in some unionized form. All in-

[2] When defibrinated blood is used, it should be remembered that the concentration of the sodium ion in the serum is determined. Since there is less sodium in the corpuscles than in plasma, results by quantitative analysis would be lower than those calculated from the electrometric determination. Incidentally a combination of the electrometric determination and quantitative analysis would indicate any abnormal distribution of sodium between corpuscles and serum.

vestigators have agreed that only a portion of the calcium is
free. Rona and Takahasi (18) found by dialysis that 75 per cent
of the Ca is diffusible. Cushny found that 60 to 70 per cent of
the Ca is filterable, while Richter-Quittner (3) who filtered at even
lower pressures found only 5 to 7 per cent of the total calcium was
filtered. The results in the table are apparently midway between
the results of Cushny and Richter-Quittner. From the results of
the table it is apparent that there are no grounds for assuming
the existence of any ion-protein combination for sodium or
chlorine, but that a calcium-protein compound is very probable.

SUMMARY.

The sodium, chloride, and calcium ion concentrations in blood
have been determined by suitable electrodes. A comparison of
the ionic concentrations found with the total concentrations as
determined by ordinary analytical methods indicate that the
sodium and chloride are present as in an aqueous solution of sodium
chloride (and sodium bicarbonate) of the same concentration,
while only about 10 per cent of the total calcium is present in
ionic form.

BIBLIOGRAPHY.

1. Burian, R., *Arch. fisiol.*, 1909, vii, 421.
2. Cushny, A. R., *J. Physiol.*, 1919–20, liii, 391.
3. Richter-Quittner, M., *Biochem. Z.*, 1921, cxxiv, 106; 1921–22, cxxvi, 97.
4. Neuhausen, B. S., *J. Am. Chem. Soc.*, 1922, xliv, 1411.
5. Lewis, G. N., and Kraus, C. A., *J. Am. Chem. Soc.*, 1910, xxxii, 1459.
6. Walker, J., and Cormack, W., *J. Chem. Soc.*, 1900, lxxvii, 5.
7. Brown, W. E. L., and Hill, A. V., *J. Physiol.*, 1920–21, liv, pp. cix and cxxi.
8. Neuhausen, B. S., *J. Am. Chem. Soc.*, 1922, xliv, 1445.
9. Neuhausen, B. S., *J. Biol. Chem.*, 1922, li, 435.
10. Rona, P., and György, P., *Biochem. Z.*, 1913, lvi, 416.
11. Hamburger, H. J., *Arch. Physiol.*, 1898, 1.
12. Evans, C. L., *J. Physiol.*, 1921, lv, 165.
13. Falta, W., and Richter-Quittner, M., *Biochem. Z.*, 1918, xci, 381.
14. Falta, W., and Richter-Quittner, M., *Biochem. Z.*, 1921, cxiv, 310.
15. van Creveld, S., *Biochem. Z.*, 1921, cxxiii, 304.
16. Rona, P., *Biochem. Z.*, 1910, xxix, 501.
17. Asher, L., and Rosenfeld, R., *Biochem. Z.*, 1907, iii, 335.
18. Rona, P., and Takahashi, D., *Biochem. Z.*, 1911, xxxi, 336.

A NOTE ON THE DETERMINATION OF URIC ACID.

By HENRY JACKSON, Jr., and WALTER W. PALMER.

(From The Department of Medicine of The College of Physicians and Sur-
geons, Columbia University, and the Presbyterian Hospital, New York.)

In a recent paper[1] we described a reagent which under given
conditions would give, with uric acid a far greater color than was
developed by the older Folin reagent. The preparation of this
reagent was time-consuming and somewhat complicated.

We have found a very simple method by which the same result
can be obtained. The "B" salt is prepared as described in the
previous paper. This dry solid is dissolved in 95 per cent alcohol
(about 250 cc. of alcohol for each 100 gm. of solid). A residue of
simple phosphates remains undissolved. The solution is filtered,
(more alcohol may be added if the solution does not filter readily),
and evaporated to dryness in a water bath, with frequent
decolorizations with bromine water. The dry product is dissolved
in a little hot water, decolorized again, and evaporated once more
to dryness.

A 20 per cent solution is now made of this purified "B" solid—
accurate to about 1 per cent. To each 100 cc. of this solution
are added 34 cc. of an exactly 2.5 per cent water solution of
primary calcium phosphate (Baker analyzed c. p.).

This final solution may now be used in the same manner as the
more complicated reagent previously described.

The same turbidity will develop in the final colored solution
and will clear up in the course of a few minutes, after which the
solution will remain clear for several hours. If the flocculent
precipitate does not entirely disappear, a little more 20 per cent
phosphotungstate "B," as prepared above, may be added.
If the crystalline precipitate should develop—in our experience
it has not—more calcium phosphate should be added.

The rest of the procedure is as before described.

[1] Jackson, H., Jr., and Palmer, W. W., J. Biol. Chem., 1922, l, 89.

CALCIUM AND PHOSPHORUS METABOLISM IN CHILDHOOD.*

By H. C. SHERMAN and EDITH HAWLEY.

(From the Department of Chemistry, Columbia University, New York.)

Previous publications from this laboratory (1, 2) have reviewed the literature dealing with the calcium requirement of the adult and have offered what appears to be a satisfactory estimate of the average amount of this element required for the maintenance of equilibrium in the normal man or woman: *viz.*, 0.45 gm. per day per 70 kilos of body weight.

The present paper describes experiments designed to determine the rate of storage of calcium in normal children of different ages and the nature and amount of the intake required to support optimum calcium storage in the growing child.

In the interest of brevity we omit a historical review which has been summarized elsewhere (1, 3) and any attempt to extract the data relating to calcium metabolism from the recent and rapidly growing literature of rickets and related diseases of the bones inasmuch as the present article treats only of the normal metabolism. The outstanding importance of calcium in the food requirements of growth has been strikingly demonstrated upon laboratory animals by Osborne and Mendel (4) and by McCollum, Simmonds, and Parsons (5). The results of previous investigations in which calcium storage has been measured in normal children are summarized with references in Table V. See also the

work of Hoffström (6) on the metabolism and storage of calcium in a pregnant woman.

The experiments here described were carried out in four series. The purpose of the first series was to study the relation of calcium retention to age. Twelve children from 3 to 13 years old were studied as to balance of intake and output of calcium and phosphorus during a period of 9 days. All the children were normal and received a normal mixed diet including a fixed allowance of 750 gm. of milk per child per day. This resulted in a nearly uniform calcium intake of about 1 gm. per child per day. The calcium retention varied from 0.15 to 0.62 gm. per day, increasing with the age and size of the child. Calculated to the basis of size the results show fair uniformity and indicate an average daily storage of 0.01 gm. of calcium and 0.008 gm. of phosphorus per kilo of body weight per day in normally growing children of 3 to 13 years of age. In the second series of experiments three of the children who had served as subjects during the first series were kept under continuous control and observation with quantitative determination of intake and output of calcium and phosphorus for 48 days—a series of eight experiments of 6 days each—the calcium intake being varied from period to period by systematic changes in the amount of milk in the diet, in order to determine what daily allowance of milk would induce optimum storage of calcium in the growing organism of the child. The three children studied in this series were 4, 5, and 12 years of age, and in each case it was found that optimum storage of calcium required an allowance of about a quart (750 or 1,000 gm.) of milk per child per day. Combining the data of both series it is found that the average storage of calcium was 70 per cent higher (0.017 gm. as against 0.010 gm. per kilo of body weight per day) when the daily allowance of milk was increased from 750 to 1,000 gm. per child per day.

When the food intake included 1,000 gm. of milk per day, the other foods of the mixed diet being taken *ad libitum*, the daily intake of calcium by these normally growing children of 4 to 12 years averaged 0.053 gm. of calcium per kilo of body weight, and of this intake approximately one-third was retained in the growing body.

The experiments of the third and fourth series were designed to determine whether children utilize the calcium of vegetables as

well as they do that of milk.   In Series III the same three children
were used as in Series II and calcium and phosphorus balances
were determined continuously for 27 days divided into three experi-
ments of 9 days each.   During the first and third of these periods
each child received 500 gm. of milk per day as the only calcium-
rich food of a mixed diet, while during the second period there were
added to the diet such amounts of carrots and spinach as would
furnish the quantity of calcium which would have been supplied
by a second 500 gm. of milk.   Had the calcium of the vegetables
been utilized as efficiently as that of milk, this would have been
equivalent to increasing the daily allowance of milk from
500 to 1,000 gm. and a distinct increase of calcium storage
would have resulted as shown by the experiments of Series
II.   This was found not to be the case.   In the final series
of experiments three different children were used and the
method of comparing the utilization of the calcium of the
vegetables with that of milk was modified by using 750 gm.
of milk per day in the first and third period while during the
second period one-half of this milk was replaced by enough
of the vegetables to furnish the same amount of calcium.   Here
again it was found that the children utilized the calcium of milk
to better advantage than they did the calcium of the vegetables.
The writers entertain no doubt as to the desirability of a liberal
use of vegetables in the feeding of children, but the vegetables
should be used in addition to a liberal allowance of milk and should
not be allowed to reduce the amount of milk consumed.

EXPERIMENTAL DATA AND DISCUSSION.

First Series.

The purpose of this series of experiments was to obtain data
which in conjunction with the data already on record would serve
to establish quantitatively the extent to which calcium is stored
in the bodies of healthy growing children of different ages under
ordinary normal conditions of diet.   Twelve healthy, well devel-
oped children from 3 to 13 years of age were studied.   Inasmuch
as the data recorded by previous investigators were chiefly for
boys (7, 8), a larger number of girls than of boys were included in
the present study.   The twelve children were divided into three
groups.

Group I consisted of four children 3 to 6 years of age. Their diet was milk, bread, butter, oatmeal, orange juice, and potato.

Group II comprised four girls 6 to 10 years old, who received milk, bread, butter, potato, oatmeal or macaroni, orange juice, and apple. Small portions of ice-cream were also given; once during the first, and once during the third period.

Group III consisted of four girls 10 to 13 years old. The diet for this group was milk, bread, butter, oatmeal, orange juice, apple, potato, and beef.

As the milk was the chief source of calcium in all cases (as in most dietaries), and it was desired to insure a fairly regular intake of calcium at about the level to which the children were accustomed, the daily allowance of milk was fixed at 750 gm. each for ten of the children; 600 and 500 gm., respectively, for the remaining two. The rest of the diet, which consisted of foods of much lower calcium content, was, in general, partaken of according to the taste and appetite of the individual.

Table I shows the average food intake of each child in grams per day of the food as served at the table. All food was weighed, sampled, and analyzed for calcium and phosphorus. The energy and protein values of the food were calculated from the values given in Rose's Laboratory Handbook for Dietetics.

The children (in groups of four as indicated above) were housed throughout the experimental periods in rooms devoted exclusively to this purpose where they were under the constant supervision and care of one of the writers (E. H.) for 1 preliminary day and 9 consecutive experimental days. The 9 experimental days were divided into three periods of 3 days each. The urine and feces of each child were collected quantitatively for each period (carmine being used to mark the beginning and end of each period in the feces), and were subsequently analyzed for calcium by the McCrudden method and for phosphorus by the gravimetric method of double precipitation first as ammonium phosphomolybdate and subsequently as magnesium ammonium phosphate.

Table II shows in grams per day the average intake, output, and balance of calcium and phosphorus for each child during each 3 day period, and Table III shows the daily intake, output, and balance for each child as averaged for the 9 experimental days treated as one period. As the balances for the successive periods

show no distinct trend upward or downward, it appears probable that calcium intakes obtaining in these experiments were in general not very different from those to which the children were accustomed; that such fluctuations from period to period as are shown by the data of Table II in the cases of some individual children are most probably due to chance variations of output; and that average balances shown by Table III afford the best available indication of the amounts of calcium and phosphorus ordinarily stored in normally growing children of 3 to 13 years of age.

It will be seen that with calcium intakes of 0.65 to 1.02 gm. per day, the balances ranged from +0.15 to +0.62 gm. per day. The amount stored does not in these cases run parallel with the intake, as between the different children, but shows a closer relationship to the age and size of the child. In the case of phosphorus the balance ranges from +0.09 gm. on an intake of 0.80 gm., to +0.53 gm. on an intake of 1.46 gm., the storage here showing a direct relationship both to the intake and to the body weight.

Table IV shows the average daily storage of calcium and of phosphorus by each child calculated in terms of body weight. It will be seen that under the dietary conditions of these experiments the children of all ages from 3 to 13 years show a fairly constant storage of about 0.01 gm. of calcium per kilo of body weight per day; while the storage of phosphorus averages 0.008 gm. per kilo of body weight, but with much larger variations among the individual children.

In Table V are summarized the data of the present series and of all similar determinations of calcium and phosphorus balances of normal children which we have found in the literature, the data being calculated to grams of element stored per day per kilo of body weight and averaged in groups according to age. From these data it appears that normal storage per kilo of body weight is highest in the second half of the first year, when it averages nearly 0.04 gm. of calcium and nearly 0.03 gm. of phosphorus; at the age of 3 to 8 years the average rate of storage has become about 0.01 gm. each of calcium and phosphorus per kilo of body weight per day and continues at nearly this rate throughout the following years of rapid growth and development.

TABLE I

Average Daily Food Intake of Each Child in Grams.

Name	Age yr.	Age m.	Weight	Milk	Bread	Butter	Orange juice.	Sugar	Oatmeal	Potato	Apple	Ice-cream.	Calories.	Protein.
			kg.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.		gm.
Period I.														
K. C.	3	7	16.2	750	198	16.5	83	8	190	117			1,402	51
C. B.	3	8	15.2	757	129	7.7	55	3	110	100			1,074	42
M. O.	4	7	19.5	736	175	15.3	83	9	167	133			1,319	48
A. B.	6		21.4	750	265	16.7	83	10	300	167			1,678	61
M. P.	6	4	19.1	602	196	16.7	52	5	18	150	46	26	1,240	42
R. G.	7	9	25.2	755	127	15.6	55	10	100	133	101	22	1,244	43
R. B.	8	6	39.3	557	163	16.7	55	3	50	200	80	28	1,198	40
R. T.	9		33.0	702	121	9.7	56	3	50	167	107	30	1,137	40
												Meat.		
L. M.	10	11	35.4	750	118	26.6	82	10	167	273	191	67	2,412	57
A. F.	11	5	31.2	749	217	26.6	82	10	216	256	187	62	1,991	72
E. C.	11	7	30.7	750	302	26.6	82	10	83	256	172	63	2,039	73
L. G.	13	6	51.9	750	510	26.6	79	3	17	273	166	62	2,501	91
Period II.														
K. C.	3	7	16.2	750	150	13.5	85	8	200	128			1,269	47
C. B.	3	8	15.2	719	245	1.0			150	67			1,296	53
M. O.	4		19.5	750	245	15.0	57	9	167	83			1,469	51
A. B.	6	7	21.4	750	264	15.0	85	10	417	150			1,731	64
									Macaroni.					
M. P.	6		19.1	600	216	26.7	78	30	131	200	1		1,681	52
R. G.	7	4	25.2	733	175	18.7	82	30	141	133	126		1,635	53

R. B.	8	9	39.3	467	162	17.7	82	30	140	123	131		1,455	43
R. T.	9	6	33.0	733	195	15.2	82	30	157	199	157		1,781	59
									Oatmeal.			Meat.		
L. M.	10	11	35.4	750	492	28.7	80	25	233	190	136	97	2,658	100
A. F.	11	5	34.2	750	383	30.3	83	35	267	190	176	95	2,439	91
E. C.	11	7	30.7	750	435	28.7	83	35	200	190	130	97	2,486	91
L. G.	13	6	54.9	750	809	28.7	83	35		190	132	97	3,293	123

Period III.

									Macaroni.					
K. C.	3	7	16.2	750	223	15.0	85	8	267	150			1,525	55
C. B.	3	8	15.2	750	231	1.0	24	5	283	117			1,396	56
M. O.	4	7	19.5	750	309	15.0	85	9	283	150			1,761	64
A. B.	6		21.4	750	357	15.0	85	10	383	200			1,976	72
												Ice-cream.		
M. P.	6	1	19.1	600	182	23.3	76	0	169	89	53	26	1,528	52
R. G.	7	9	25.3	748	196	23.3	80	0	171	150	117	27	1,753	39
R. B.	8	6	39.3	500	279	23.3	80	0	199	196	117	30	1,868	61
R. T.	9		33.0	750	311	25.0	80	0	187	238	88	29	2,197	73
									Oatmeal.			Meat.		
L. M.	10	11	35.4	715	485	31.7	82	10	200	210	152	97	2,672	100
A. F.	11	5	31.2	750	361	33.0	82	10	300	210	175	97	2,147	91
E. C.	11	7	30.7	750	454	31.7	82	10	200	223	156	97	2,512	95
L. G.	13	6	51.9	750	761	33.3	82	0		223	141	97	3,236	119

TABLE II.

Calcium and Phosphorus Storage during First Series of Experiments, in Grams per Day.

Name	Sex	Age		Weight	Calcium				Phosphorus			
					In-take.	Urine.	Feces.	Balance.	In-take.	Urine.	Feces.	Balance.
		yr.	mo.	kg.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.

Period I.

Name	Sex	Age		Weight	In-take.	Urine.	Feces.	Balance.	In-take.	Urine.	Feces.	Balance.
K. C.	F.	3	7	16.2	0.883	0.025	0.775	+0.084	1.029	0.531	0.407	+0.091
C. B.	M.	3	8	15.2	0.859	0.015	0.696	+0.148	0.917	0.465	0.326	+0.125
M. O.	F.	4	7	19.5	0.862	0.010	0.598	+0.254	0.990	0.497	0.388	+0.105
A. B.	M.	6		21.4	0.914	0.107	0.628	+0.179	1.168	0.668	0.432	+0.068
M. P.	F.	6		19.1	0.738	0.048	0.413	+0.277	0.827	0.613	0.204	+0.010
R. G.	"	7	4	25.2	0.892	0.051	0.544	+0.297	0.955	0.500	0.343	+0.112
R. B.	"	8	9	39.3	0.693	0.015	0.288	+0.390	0.800	0.532	0.168	+0.100
R. T.	"	9	6	33.0	0.837	0.043	0.518	+0.276	0.889	0.517	0.316	+0.056
L. M.	"	10	11	35.4	0.976	0.072	0.605	+0.299	1.325	0.682	0.407	+0.236
A. F.	"	11	5	34.2	0.940	0.026	0.557	+0.357	1.205	0.653	0.461	+0.091
E. C.	"	11	7	30.7	0.939	0.032	0.459	+0.448	1.173	0.594	0.329	+0.250
L. G.	"	13	6	54.9	0.979	0.033	0.470	+0.476	1.311	0.448	0.355	+0.508

Period II.

Name	Sex	Age		Weight	In-take.	Urine.	Feces.	Balance.	In-take.	Urine.	Feces.	Balance.
K. C.	F.	3	7	16.2	0.873	0.016	0.733	+0.124	0.999	0.578	0.376	+0.045
C. B.	M.	3	8	15.2	0.872	0.028	0.732	+0.112	1.008	0.535	0.332	+0.141
M. O.	F.	4	7	19.5	0.886	0.011	0.740	+0.135	1.038	0.506	0.419	+0.113
A. B.	M.	6		21.4	0.925	0.108	0.573	+0.244	1.231	0.658	0.456	+0.117
M. P.	F.	6		19.1	0.734	0.024	0.522	+0.188	0.884	0.475	0.222	+0.187
R. G.	"	7	4	25.2	0.871	0.055	0.617	+0.199	0.968	0.572	0.361	+0.035
R. B.	"	8	9	39.3	0.582	0.029	0.370	+0.183	0.708	0.520	0.202	−0.013
R. T.	"	9	6	33.0	0.881	0.039	0.631	+0.211	1.016	0.449	0.408	+0.159
L. M.	"	10	11	35.4	0.991	0.093	0.601	+0.297	1.419	0.526	0.441	+0.452
A. F.	"	11	5	34.2	0.974	0.044	0.696	+0.234	1.353	0.473	0.444	+0.436
E. C.	"	11	7	30.7	0.975	0.051	0.514	+0.410	1.350	0.527	0.353	+0.470
L. G.	"	13	6	54.9	1.039	0.068	0.261	+0.710	1.547	0.602	0.226	+0.719

Period III.

Name	Sex	Age		Weight	In-take.	Urine.	Feces.	Balance.	In-take.	Urine.	Feces.	Balance.
K. C.	F.	3	7	16.2	0.899	0.013	0.540	+0.346	1.107	0.521	0.319	+0.267
C. B.	M.	3	8	15.2	0.891	0.028	0.683	+0.180	1.100	0.532	0.355	+0.213
M. O.	F.	4	7	19.5	0.921	0.015	0.748	+0.158	1.190	0.418	0.519	+0.253
A. B.	M.	6		21.4	0.946	0.126	0.621	+0.199	1.306	0.707	0.462	+0.137
M. P.	F.	6		19.1	0.751	0.030	0.497	+0.224	0.839	0.537	0.182	+0.120
R. G.	"	7	4	25.2	0.920	0.066	0.483	+0.371	1.017	0.515	0.254	+0.247
R. B.	"	8	9	39.3	0.687	0.018	0.373	+0.296	0.887	0.442	0.251	+0.193
R. T.	"	9	6	33.0	0.965	0.043	0.590	+0.332	1.176	0.393	0.406	+0.377
L. M.	"	10	11	35.4	0.985	0.056	0.618	+0.311	1.408	0.771	0.409	+0.228
A. F.	"	11	5	34.2	0.974	0.028	0.607	+0.339	1.371	0.640	0.398	+0.333
E. C.	"	11	7	30.7	0.979	0.043	0.526	+0.410	1.365	0.711	0.322	+0.332
L. G.	"	13	6	54.9	1.032	0.116	0.237	+0.679	1.522	0.992	0.175	+0.355

TABLE III

Average Calcium and Phosphorus Storage for the 9 Days of the First Series, in Grams per Day.

Name.	Age.		Weight.	Calcium.			Phosphorus.		
				Intake	Output.	Balance.	Intake	Output.	Balance.
	yr.	mo.	kg.	gm.	gm.	gm.	gm.	gm.	gm.
K. C.	3	7	16.2	0.885	0.700	+0.185	1.045	0.910	+0.135
C. B.	3	8	15.2	0.874	0.727	+0.147	1.009	0.848	+0.161
M. O.	4	7	19.5	0.890	0.707	+0.183	1.073	0.916	+0.157
A. B.	6		21.4	0.928	0.721	+0.207	1.235	1.128	+0.107
M. P.	6		19.1	0.741	0.511	+0.230	0.850	0.744	+0.106
R. G.	7	4	25.2	0.894	0.605	+0.289	0.980	0.848	+0.132
R. B.	8	9	39.3	0.654	0.364	+0.290	0.798	0.705	+0.093
R. T.	9	6	33.0	0.894	0.621	+0.273	1.028	0.830	+0.198
L. M.	10	11	35.4	0.984	0.682	+0.302	1.384	1.079	+0.305
A. F.	11	5	34.2	0.963	0.653	+0.310	1.310	1.023	+0.287
E. C.	11	7	30.7	0.964	0.541	+0.423	1.296	0.946	+0.350
L. G.	13	6	54.9	1.017	0.395	+0.622	1.460	0.933	+0.527

TABLE IV.

Calcium and Phosphorus Storage According to Weight, in Grams per Kilo per Day.

Name.	Age.		Weight.	Calories.	Protein.	Calcium.			Phosphorus.		
						In-take.	Out-put.	Balance.	In-take	Out-put	Balance.
	yr.	mo.	kg.		gm.	gm.	gm.	gm.	gm.	gm.	gm.
K. C.	3	7	16.2	86	3.1	0.054	0.043	+0.011	0.062	0.054	+0.008
C. B.	3	8	15.2	83	3.3	0.058	0.048	+0.010	0.067	0.056	+0.011
M. O.	4	7	19.5	78	2.8	0.046	0.037	+0.009	0.055	0.047	+0.008
A. B.	6		21.4	84	3.1	0.043	0.033	+0.010	0.058	0.053	+0.005
M. P.	6		19.1	78	2.6	0.039	0.027	+0.012	0.045	0.039	+0.006
R. G.	7	4	25.2	61	2.1	0.035	0.024	+0.011	0.039	0.034	+0.005
R. B.	8	9	39.3	38	1.2	0.017	0.010	+0.007	0.020	0.018	+0.002
R. T.	9	6	33.0	52	1.8	0.027	0.019	+0.008	0.031	0.025	+0.006
L. M.	10	11	35.4	73	2.7	0.028	0.019	+0.009	0.039	0.030	+0.009
A. F.	11	5	34.2	67	2.5	0.028	0.019	+0.009	0.038	0.030	+0.008
E. C.	11	7	30.7	77	2.9	0.031	0.017	+0.014	0.042	0.031	+0.011
L. G.	13	6	54.9	55	2.0	0.018	0.007	+0.011	0.027	0.017	+0.010

TABLE V.

Average Calcium and Phosphorus Storage of Children, in Grams per Kilo per Day.

Age.	No. of cases	Calcium.			No. of cases	Phosphorus.			References.
		In-take.	Out-put.	Balance.		In-take.	Out-put.	Balance.	
		gm.	*gm.*	*gm.*		*gm.*	*gm.*	*gm.*	
1–6 mo.	32	0.090	0.063	+0.027	11	0.075	0.058	+0.017	(10–20, 25, 27)
7–12 mo.	14	0.137	0.098	+0.039	5	0.115	0.087	+0.028	(9, 12, 16, 18, 21–23, 25, 28)
1–2 yrs.	5	0.089	0.070	+0.019					(12)
3–8 yrs.	13	0.039	0.028	+0.011	10	0.054	0.046	+0.008	(7, 26, 27, Table IV above)
9–14 yrs.	14	0.027	0.018	+0.009	11	0.039	0.029	+0.010	(7, 8, 24, Table IV above)

Second Series.

This series of experiments was an intensive study of the calcium metabolism of three children to determine on what amount of calcium they made optimum storage. Milk was again used as the chief source of calcium, and the variations of calcium intake were accomplished by regularly graduated alterations in the daily allowance of milk. The series consisted of eight consecutive experiments of 6 days each. During the first experiment each child received 250 gm. of milk per day; during the second, 500 gm.; the third, 750 gm.; the fourth, 1,000 gm.; the fifth, 1,500 gm.; the sixth, 1,000 gm.; the seventh, 750 gm.; the eighth, 500 gm. per day. Each 6 day experiment was divided into two 3 day periods for sampling and analysis of food and collection and analysis of urine and feces. The first 3 days of each experiment were intended to allow for adjustment to the diet and the second 3 day period to show how much storage the diet would induce.

Experience showed, however, that it was at least equally satisfactory to use the average of all 6 days. In addition to milk, the diet used in this series of experiments furnished bread, butter, orange juice, and oatmeal daily with potato and macaroni in alternate 3 day periods. Apple sauce, corn flakes, and prunes were also used. The amount of each food consumed by each child is

TABLE VI.

Average Daily Food Intake During the Second Series of Experiments, in Grams per Day.

Experiment No.	I	II	III	IV	V	VI	VII	VIII
E. C., female, age 12 years; weight 33.4 to 35.4 kilos.								
	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.
Milk	250	500	750	1,000	1,500	1,000	750	500
Bread	288	440	406	382	370	325	381	334
Butter	30	30	30	30	30	30	30	30
Orange juice	60	75	85	69	58	60	60	64
Oatmeal	200	100	167	150	67	150	117	100
Potato	459		400		388		442	
Macaroni		369		394		460		463
Cocoa	180	187						175
Apple sauce	134	275						
Corn flakes			50	47	44	50		
Prunes							100	100
Sugar	45	14	6	8	8	10	12	16
Strawberries							33	3
Cream of wheat		17						
Calories	1,863	2,784	2,357	2,946	2,678	2,936	2,274	2,634
Protein	51	90	77	103	94	101	71	86
Calcium	0.425	0.748	0.994	1.273	1.794	1.262	1.015	0.741
Phosphorus	0.886	1.211	1.460	1.619	2.009	1.559	1.367	1.128
M. O., female, age 5 years, 2 months; weight 20.2 to 22.1 kilos.								
Milk	250	500	750	1,000	1,500	1,000	750	500
Bread	224	303	243	284	182	175	281	277
Butter	28	25	25	25	25	25	25	25
Orange juice	60	58	60	62	58	60	60	64
Oatmeal	150	75	142			100	100	100
Potato	242		150		134		142	
Macaroni		157		188		215		167
Cocoa	117	134						200
Apple sauce	125	150						
Corn flakes				30	30	30	29	
Prunes							64	60
Sugar	35	10	10	5	5	10	11	10
Strawberries							19	
Cream of wheat		67						
Calories	1,469	1,932	1,687	2,112	1,922	1,960	1,753	1,844
Protein	39	61	56	74	71	68	57	60
Calcium	0.386	0.682	0.930	1.208	1.727	1.196	0.954	0.686
Phosphorus	0.697				1.675		1.133	0.950

TABLE VI—*Concluded*.

Experiment No.	I	II	III	IV	V	VI	VII	VIII
K. C., female, age 4 years, 2 months; weight 17.0 to 18.6 kilos.								
	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.
Milk	250	500	750	1,000	1,500	1,000	750	500
Bread	222	319	235	217	101	161	237	200
Butter	28	25	25	25	23	25	25	25
Orange juice	60	58	60	62	58	60	60	64
Oatmeal	142	75	150	150	75	84	100	100
Potato	259		200		142		150	
Macaroni		117		142		72		142
Cocoa	125	134						200
Apple sauce	100							
Corn flakes			30	30	24	29		
Prunes							52	52
Sugar	38	10	10	10	7	9	12	13
Strawberries							19	
Cream of wheat		67						
Calories	1,341	1,812	1,690	1,976	1,761	1,653	1,627	1,608
Protein	40	60	56	68	65	57	53	52
Calcium	0.386	0.671	0.933	1.206	1.717	1.177	0.942	0.665
Phosphorus	0.695	0.930	1.167	1.343		1.198		0.863

shown in Table VI. The technique of weighing, sampling, and analysis of intake and output was similar to that described for the first series and three of the same children, aged 4, 5, and 12 years, served as subjects.

Tables VII and VIII show the results of these experiments, averaged both for 3 day and for 6 day periods.

The data show that E. C. (12 years old) stored only 0.007 gm. of calcium per kilo of body weight when receiving 250 gm. of milk per day. As the intake of milk was increased there was increased retention of calcium up to 0.022 gm. per kilo with 1,000 gm. of milk and 0.023 gm. per kilo with 1,500 gm. of milk. Examination of the results obtained with K. C. (age 4 years) shows the same response to increased intake except that the differences in amounts of calcium stored were not so great. The results with M. O. (age 5 years) were less regular and the storage of calcium was lower throughout. The results of the series as a whole indicate 1,000 gm. of milk per day as the most probable intake required to support an optimum storage of calcium in the body.

TABLE VII.

Calcium and Phosphorus Storage on Varying Amounts of Milk (Second Series), in Grams per Day.

Experiment No.	Calcium.				Phosphorus.			
	Intake.	Urine.	Feces.	Balance	Intake.	Urine.	Feces.	Balance.

E. C., female, age 12 years; weight 33.4 to 35.4 kilos.

Experiment No.	Intake (gm.)	Urine (gm.)	Feces (gm.)	Balance (gm.)	Intake (gm.)	Urine (gm.)	Feces (gm.)	Balance (gm.)
I, 1	0.425	0.026	0.204	+0.195	0.893	0.438	0.280	+0.175
2	0.425	0.018	0.158	+0.249	0.879	0.487	0.236	+0.156
II, 1	0.731	0.034	0.240	+0.457	1.124	0.650	0.230	+0.244
2	0.764	0.033	0.277	+0.454	1.297	0.371	0.357	+0.569
III, 1	0.981	0.021	0.230	+0.730	1.398	0.549	0.199	+0.650
2	1.006	0.033	0.245	+0.727	1.522	0.584	0.258	+0.680
IV, 1	1.272	0.087	0.432	+0.753	1.604	0.714	0.304	+0.586
2	1.273	0.102	0.361	+0.810	1.634	0.750	0.272	+0.612
V, 1	1.803	0.119	0.799	+0.885	2.082	1.193	0.442	+0.447
2	1.785	0.127	0.892	+0.766	1.935	1.140	0.329	+0.466
VI, 1	1.265	0.129	0.516	+0.620	1.570	0.913	0.338	+0.289
2	1.259	0.178	0.429	+0.652	1.547	0.881	0.278	+0.388
VII, 1	1.012	0.126	0.438	+0.448	1.352	0.928	0.289	+0.135
2	1.019	0.099	0.281	+0.639	1.382	0.883	0.191	+0.308
VIII, 1	0.746	0.102	0.238	+0.406	1.146	0.536	0.199	+0.411
2	0.735	0.121	0.215	+0.399	1.110	0.529	0.195	+0.386

M. O., female, age 5 years, 2 months; weight 20.2 to 22.1 kilos.

Experiment No.	Intake	Urine	Feces	Balance	Intake	Urine	Feces	Balance
I, 1	0.381	0.023	0.284	+0.074	0.675	0.334	0.289	+0.052
2	0.392	0.016	0.253	+0.123	0.719	0.325	0.256	+0.138
II, 1	0.679	0.016	0.472	+0.191	0.920	0.369	0.308	+0.243
2	0.684	0.019	0.417	+0.248				
III, 1	0.929	0.025	0.713	+0.191				
2	0.931	0.022	0.704	+0.205				
IV, 1	1.207	0.027	0.905	+0.275				
2	1.209	0.023	0.949	+0.237				
V, 1	1.735	0.033	1.494	+0.208	1.730	0.658	0.769	+0.303
2	1.719	0.021	1.473	+0.225	1.620	0.699	0.737	+0.184
VI, 1	1.196	0.022	0.863	+0.311	1.278	0.578	0.474	+0.226
2	1.195	0.026	0.895	+0.274				
VII, 1	0.952	0.022	0.730	+0.200	1.119	0.523	0.359	+0.237
2	0.955	0.025	0.786	+0.144	1.147	0.529	0.415	+0.203
VIII, 1	0.700	0.034	0.565	+0.101	1.014	0.436	0.321	+0.257
2	0.671	0.025	0.504	+0.142	0.886	0.450	0.261	+0.175

TABLE VII—*Concluded*.

Experiment No	Calcium.				Phosphorus.			
	Intake	Urine.	Feces.	Balance	Intake	Urine.	Feces.	Balance.
K. C., female, age 4 years, 2 months; weight 17.0 to 18.6 kilos.								
	gm.	*gm.*	*gm.*	*gm*	*gm.*	*gm.*	*gm.*	*gm.*
I, 1	0.386	0.019	0.180	+0.187	0.688	0.239	0.168	+0.281
2	0.385	0.010	0.203	+0.172	0.701	0.283	0.180	+0.238
II, 1	0.668	0.027	0.321	+0.320	0.894	0.379	0.185	+0.330
2	0.674	0.029	0.453	+0.192	0.965	0.447	0.249	+0.269
III, 1	0.931	0.037	0.562	+0.332	1.159	0.539	0.266	+0.354
2	0.934	0.031	0.667	+0.236	1.175	0.543	0.282	+0.350
IV, 1	1.210	0.049	0.858	+0.303	1.351	0.780	0.333	+0.238
2	1.202	0.037	0.886	+0.279	1.334	0.669	0.393	+0.272
V, 1	1.731	0.046	1.449	+0.236	1.733	0.759	0.698	+0.276
2	1.702	0.025	1.290	+0.387				
VI, 1	1.174	0.035	0.939	+0.200	1.186	0.606	0.376	+0.204
2	1.179	0.021	0.836	+0.322	1.210	0.553	0.322	+0.335
VII, 1	0.940	0.035	0.592	+0 313	1.077	0.509	0.241	+0.327
2	0.943	0.044	0.678	+0.221				
VIII, 1	0.670	0.034	0.409	+0.227	0.891	0.541	0.190	+0.160
2	0.659	0.034	0.440	+0.185	0.834	0.500	0.210	+0.124

Third Series.

The experiments of this series were undertaken to determine whether children could utilize calcium in the form of vegetables as efficiently as they had utilized the calcium of milk in the experiments described above. The three children who had been used in the second series served again as subjects.

There were three consecutive experiments of 9 days each, this longer time being allowed in order to provide ample opportunity for adjustment to the dietary changes involved. Each child received throughout these experiments 500 gm. of milk per day with bread, butter, oatmeal, and orange juice. During the first 9 days they received a large amount of potato, selected as a familiar and acceptable vegetable of low calcium content. During the second 9 days they received, instead of the potato, carrots and spinach in such amount as to make the total calcium of the diet the same as that of the diet containing 1,000 gm. of milk which in the second series had induced optimun storage. During the last 9 days the diet was the same as during the first. Each of the 9

day experiments was divided into three 3 day periods for collection and analysis of foods, feces, and urine.

TABLE VIII.

Average Calcium and Phosphorus Balances for the Second Series of Experiments, in Grams per Day.

Experiment No.	Calcium.				Phosphorus.			
	Intake.	Output.	Balance	Balance per kilo	Intake.	Output.	Balance.	Balance per kilo
E. C., female, age 12 years; weight 33.2 to 35.4 kilos.								
	gm.	gm.	gm.	gm	gm.	gm	gm	gm.
I	0.425	0.203	+0.222	+0.007	0.886	0.721	+0.165	+0.005
II	0.748	0.292	+0.456	+0.013	1.211	0.804	+0.407	+0.012
III	0.994	0.265	+0.739	+0.021	1.460	0.795	+0.665	+0.019
IV	1.273	0.491	+0.782	+0.022	1.619	1.020	+0.599	+0.017
V	1.794	0.969	+0.825	+0.023	2.009	1.552	+0.457	+0.013
VI	1.262	0.626	+0.636	+0.018	1.559	1.220	+0.339	+0.010
VII	1.015	0.472	+0.544	+0.015	1.367	1.146	+0.221	+0.006
VIII	0.741	0.338	+0.403	+0.011	1.128	0.730	+0.398	+0.011
M. O., female, age 5 years, 2 months; weight 20.2 to 22.1 kilos.								
I	0.386	0.288	+0.098	+0.005	0.697	0.602	+0.095	+0.005
II	0.682	0.462	+0.220	+0.011				
III	0.930	0.732	+0.198	+0.009				
IV	1.208	0.952	+0.256	+0.012				
V	1.727	1.510	+0.217	+0.010	1.675	1.432	+0.243	+0.011
VI	1.196	0.903	+0.293	+0.014				
VII	0.954	0.782	+0.172	+0.008	1.133	0.913	+0.220	+0.010
VIII	0.686	0.564	+0.122	+0.006	0.950	0.734	+0.216	+0.010
K. C., female, age 4 years, 2 months; weight 17.0 to 18.6 kilos.								
I	0.386	0.206	+0.180	+0.011	0.695	0.435	+0.260	+0.015
II	0.671	0.415	+0.256	+0.015	0.930	0.630	+0.300	+0.017
III	0.933	0.649	+0.284	+0.016	1.167	0.815	+0.352	+0.020
IV	1.206	0.915	+0.291	+0.016	1.343	1.088	+0.255	+0.014
V	1.717	1.405	+0.312	+0.017				
VI	1.177	0.916	+0.261	+0.015	1.198	0.929	+0.269	+0.015
VII	0.942	0.675	+0.267	+0.015				
VIII	0.665	0.459	+0.206	+0.011	0.863	0.721	+0.142	+0.008

Tables IX, X, and XI give the detailed data of these experiments presented in the same general manner as in the previous series.

TABLE IX

Average Daily Food Intake during the Third Series of Experiments, in Grams per Day.

Food.	Experiment I.			Experiment II.			Experiment III.		
	1	2	3	1	2	3	1	2	3

E. C., female, age 12 years, 9 months; weight 36.6 to 38.4 kilos.

Food.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.
Milk	500	500	500	500	500	500	500	500	500
Bread	317	474	579	633	593	598	567	500	467
Butter	30	30	30	30	30	30	30	30	30
Orange juice	73	80	80	80	80	80	80	80	80
Oatmeal	150	150	150	150	150	150	150	150	150
Potato	483	577	600				589	600	600
Spinach				279	350	349			
Carrots				279	300	317			
French dressing				16	15	15			
Sugar	10	10	10	15	20	20	15	15	15
Calories	1,815	2,272	2,554	2,711	2,650	2,669	2,539	2,370	2,285
Protein	58	74	84	92	91	92	83	77	74
Calcium	0.687	0.740	0.774	1.201	1.267	1.258	0.769	0.750	0.740
Phosphorus	0.945	1.109	1.205	1.349	1.417	1.428	1.191	1.139	1.111

M. O., female, age 5 years, 10 months; weight 22.0 to 23.0 kilos.

Food.									
Milk	500	500	500	500	500	500	500	499	500
Bread	245	303	358	387	402	367	333	179	217
Butter	25	25	25	25	25	25	25	25	25
Orange juice	73	80	80	80	80	80	80	80	80
Oatmeal	142	150	150	150	167	167	183	83	150
Potato	417	450	450				450	433	450
Spinach				222	250	250			
Carrots				222	250	283			
French dressing				8	12	12			
Sugar	5	5	5	10	15	15	10	10	10
Calories	1,531	1,707	1,842	1,924	2,037	1,955	1,828	1,353	1,505
Protein	50	56	61	67	70	67	60	42	48
Calcium	0.659	0.679	0.696	1.033	1.071	1.056	0.691	0.639	0.653
Phosphorus	0.860	0.924	0.970	1.079	1.162	1.146	0.961	0.791	0.851

K. C., female, age 4 years, 11 months; weight 18.7 to 19.8 kilos.

Food.									
Milk	500	500	500	500	500	500	500	500	500
Bread	180	203	276	275	263	232	250	233	183
Butter	25	25	25	25	25	25	25	25	25
Orange juice	73	80	80	80	80	80	80	80	80

TABLE IX—*Concluded.*

Food.	Experiment I.			Experiment II.			Experiment III.		
	1	2	3	1	2	3	1	2	3

K. C., female, age 4 years, 11 months; weight 18.7 to 19.8 kilos—*Concluded.*

	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.
Oatmeal	142	100	117	150	150	183	200	200	200
Potato	517	483	450				467	494	517
Spinach				208	250	250			
Carrots				208	250	267			
French dressing				12	15	15			
Sugar	5	5	5	10	15	15	10	10	10
Calories	1,412	1,432	1,615	1,650	1,686	1,632	1,631	1,601	1,482
Protein	45	46	53	56	57	55	53	52	47
Calcium	0.646	0.646	0.668	0.977	1.028	1.014	0.670	0.667	0.653
Phosphorus	0.838	0.833	0.889	0.971	1.040	1.034	0.902	0.897	0.863

The results here are more variable than in either of the other series, but show in the case of each of the children studied a much lower storage of calcium on the high vegetable diet than on the diets previously used in which the same or even smaller amounts of calcium were supplied in the form of milk. The phosphorus balances are discussed in connection with those of the fourth series below.

Fourth Series.

Since the experiments of the third series were made in February and it has been found (29) that in general children do not maintain such active growth in winter as in summer, it was thought that this might perhaps have been a factor in the low rates of storage of calcium and phosphorus on the diets in which vegetables replaced half of the milk. Hence another series of experiments (fourth series) was carried out during August and September. The plan of the experiments was also modified to provide for a more direct comparison of the vegetables and milk as sources of calcium for the growing child. In this (fourth) series, 750 gm. of milk per day were given during the first 9 days; during the next 15 days the daily allowance of milk was reduced to 375 gm. and enough vegetables were added to yield an equivalent amount of calcium;

TABLE X

Calcium and Phosphorus Balances in the Experiments of the Third Series, in Grams per Day.

Experiment No		Calcium.				Phosphorus.			
		Intake.	Urine.	Feces.	Balance.	Intake	Urine.	Feces.	Balance.

E. C., female, age 12 years, 9 months; weight 36.6 to 38 4 kilos.

		gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.
I,	1	0.687	0.037	0.368	+0 282	0.945	0.557	0.263	+0.125
	2	0.740	0.036	0.550	+0.154	1.109	0.722	0.354	+0.033
	3	0.774	0.047	0.693	+0.034	1.205	0.785	0.430	−0.010
II,	1	1.201	0.065	0.801	+0.335	1.349	0.847	0.313	+0.189
	2	1.267	0.056	1.231	−0.020	1.417	0.924	0.429	+0.064
	3	1.258	0.067	1 048	+0.143	1.428	0.947	0.352	+0.129
III,	1	0.769	0.070	0.583	+0 116	1.191	0.672	0.337	+0.182
	2	0.750	0.068	0.553	+0.129	1.139	0.738	0.356	+0.045
	3	0.740	0.057	0.577	+0.106	1.111	0.592	0.362	+0.157

M. O., female, age 5 years, 10 months; weight 22.0 to 23.0 kilos.

I,	1	0.659	0.005	0.491	+0.163	0.860	0.471	0.356	+0.033
	2	0.679	0.009	0.551	+0.119	0.924	0.523	0.354	+0.047
	3	0.696	0.010	0.525	+0.161	0.970	0.587	0.346	+0.037
II,	1	1.033	0.013	0.912	+0.108	1.079	0.622	0.413	+0.044
	2	1.071	0.015	1.014	+0.042	1.162	0.639	0.414	+0.109
	3	1.056	0.016	0.962	+0 078	1.146	0.577	0.400	+0.169
III,	1	0.691	0.013	0.579	+0.099	0.961	0.459	0.427	+0.075
	2	0.639	0.010	0.460	+0.169	0.791	0.529	0.267	−0.005
	3	0.653	0.007	0.596	+0 050	0.851	0.549	0.398	−0.096

K. C., female, age 4 years, 11 months; weight 18.7 to 19.8 kilos.

I,	1	0.646	0.019	0.549	+0.078	0.838	0.483	0.332	+0.023
	2	0.646	0.016	0.502	+0 128	0.833	0.505	0.281	+0.047
	3	0.668	0.022	0.457	+0 189	0.889	0.541	0.247	+0.101
II,	1	0.977	0.016	0.785	+0.176	0.971	0.627	0.307	+0.037
	2	1.028	0.019	0.901	+0 108	1.040	0.625	0.294	+0.121
	3	1.014	0.015	0.977	+0.023	1 034	0.456	0.352	+0.226
III,	1	0.670	0.020	0.472	+0.178	0 902	0.416	0.258	+0.228
	2	0.667	0.019	0.392	+0.256	0.897	0.548	0.220	+0.129
	3	0.653	0.016	0.563	+0.074	0.863	0.510	0.320	+0.033

after which the subjects returned for a period of 6 days to the diet of the first 9 days. Carrots, spinach, and celery served as the vegetable source of calcium. The rest of the diet was similar to that used in the former experiments—bread, butter, orange juice, oatmeal, potato, and apple.

Three children served as subjects: L. M., a girl of 13 years; V.M., a girl 10 years old; and A. M. a boy 6 years old. Table XII shows the food intake of each child and Tables XIII and XIV

TABLE XI.

Average Calcium and Phasphorus Balances in the Third Series of Experiments, in Grams per Day.

Experi- ment No.	Calcium.				Phosphorus.			
	Intake.	Output.	Balance.	Balance per kilo.	Intake	Output.	Balance.	Balance per kilo.
E. C., female, age 12 years, 9 months; weight 36 6 to 38.4 kilos.								
	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.
I	0.734	0.577	+0.157	+0.004	1.086	1.037	+0 049	+0.001
II	1.242	1.090	+0.152	+0.004	1.398	1.271	+0.127	+0.003
III	0.753	0.636	+0.117	+0.003	1.147	1.019	+0.085	+0.002
M. O., female, age 5 years, 10 months; weight 22.0 to 23.0 kilos.								
I	0.678	0.530	+0.148	+0.007	0.918	0.879	+0.039	+0.002
II	1.053	0.977	+0 076	+0.003	1.129	1.022	+0.107	+0.005
III	0.661	0.555	+0.106	+0.005	0.868	0.876	−0 008	±0.000
K. C., female, age 4 years, 11 months; weight 18 7 to 19.8 kilos.								
I	0.654	0.522	+0.132	+0.007	0.853	0.796	+0.057	+0.003
II	1.006	0.904	+0.102	+0.005	1 015	0.887	+0.128	+0.007
III	0.663	0.494	+0.169	+0.009	0 887	0.757	+0.130	+0.007

show the data of intake and output of calcium and phosphorus. The second girl, V. M., did not continue during the final 6 day period of the series.

In all three cases the storage of calcium during the first 9 days approximates the average figure found in the first series of experiments, 0.01 gm. per kilo of body weight per day and in all three cases the balance became much less favorable when half of the milk was replaced by vegetables. In the case of L. M. tonsilitis

TABLE XII.

Average Daily Food Intake during the Fourth Series of Experiments, in Grams per Day.

Food	Experiment I			Experiment II.					Experiment III.	
	1	2	3	1	2	3	4	5	1	2

L. M., female, age 12 years, 9 months; weight 48 kilos.

Food	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.
Milk	750	750	749	375	375	375	375	375	750	750
Bread	405	467	500	500	520	533	400	468	452	500
Butter	40	40	40	38	40	40	40	40	40	40
Orange juice	70	83	85	85	85	85	85	85	85	85
Oatmeal	150	150	150	150	150	150	150	150	150	150
Potato	483	483	500	117	200	200	200	200	550	550
Apple	65	89	98	58	117	133	104	88	191	210
Spinach				300	330	308	263	296		
Carrots				100	100	100	100	100		
String beans				17						
Celery							40	57	50	
Sugar	8	9	35	8	8	8	8	8	8	8
Calories	2,277	2,461	2,662	2,218	2,352	2,405	2,036	2,207	2,512	2,646
Protein	73	79	82	73	76	78	65	72	79	83
Calcium	1.027	1.047	1.057	1.030	1.076	1.091	1.002	1.056	1.053	1.068
Phosphorus	1.285	1.343	1.379	1.078	1.139	1.152	1.019	1.091	1.366	1.408

V. M., female, age 10 years, 3 months; weight 32.4 to 32 kilos.

Food	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.		
Milk	750	750	750	375	375	375	375	375		
Bread	167	252	300	333	253	247	351	358		
Butter	40	40	40	38	37	38	40	40		
Orange juice	70	83	85	85	85	85	85	85		
Oatmeal	150	150	150	150	150	133	150	150		
Potato	450	450	450	117	200	100	100	100		
Apple	66	80	92	62	82	66	113	88		
Spinach				266	213	196	200	200		
Carrots				117	100	167	200	200		
String beans				28						
Celery						50	100	100		
Sugar	5	6	35	5	5	5	5	5		
Calories	1,534	1,872	2,121	1,779	1,587	1,546	1,896	1,899		
Protein	50	59	63	57	50	48	59	60		
Calcium	0.959	0.985	0.999	0.953	0.874	0.889	0.981	0.981		
Phosphorus	1.075	1.152	1.193	0.931	0.858	0.833	0.959	0.962		

TABLE XII—*Concluded*.

Food.	Experiment I.			Experiment II					Experiment III.	
	1	2	3	1	2	3	4	5	1	2

A. M., male, age 6 years, 1 month; weight 17.1 to 17.9 kilos.

Food.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
Milk	750	750	750	375	375	375	375	375	750	750
Bread	108	180	200	200	200	237	227	198	205	200
Butter	27	30	30	30	30	30	30	30	30	30
Orange juice	70	83	85	85	85	77	85	85	85	85
Oatmeal	150	150	150	150	150	150	150	150	150	150
Potato	400	400	400	117	200	200	200	200	450	450
Apple		62	69	53	59	22	55	33	51	26
Spinach				233	275	247	250	216		
Carrots				133	100	100	100	100		
String beans				17						
Celery						33	10	46		
Sugar	5	5	30	5	5	5	5	5	5	5
Calories	1,318	1,571	1,728	1,363	1,390	1,460	1,454	1,364	1,651	1,622
Protein	44	51	53	44	45	49	47	44	54	53
Calcium	0.932	0.958	0.964	0.876	0.860	0.910	0.897	0.873	0.968	0.965
Phosphorus	0.999	1.069	1.089	0.803	0.803	0.857	0.850	0 815	1.106	1.099

complicated the last 6 days (but only these) of the second period. V. M. found it so difficult to accept the high vegetable diet that her food intake was not uniform during the first half of the second period.   A. M. showed the most favorable results with the high vegetable diet but even here the substitution of vegetables for half of the milk affected the calcium balance very unfavorably.

Rose has shown (30) that it is possible to meet the calcium requirement of the human adult by the use of carrots, and somewhat similar results have been published by Blatherwick and Long (31) since the experiments here described were made. There is no doubt that the calcium of vegetables is assimilable by man and is a factor in the food value of the vegetables and one reason for including them in the diet. The experiments which we here described appear to show quite definitely that milk is much superior to vegetables as a source of calcium for the growing child. The superiority of milk to vegetables as a source of calcium for dogs had previously been shown by McClugage and Mendel (32).

Ca and P Metabolism in Childhood

TABLE XIII.

Calcium and Phosphorus Balances for the Fourth Series of Experiments, in Grams per Day.

Experiment No.		Calcium.				Phosphorus.			
		Intake.	Urine.	Feces.	Balance.	Intake.	Urine.	Feces.	Balance.

L. M., female, age 12 years, 9 months; weight 48 kilos.

Experiment No.		Intake gm.	Urine gm.	Feces gm.	Balance gm.	Intake gm.	Urine gm.	Feces gm.	Balance gm.
I,	1	1.027	0.067	0.554	+0.406	1.285	0.768	0.335	+0.182
	2	1.047	0.076	0.498	+0.473	1.343	0.822	0.292	+0.229
	3	1.057	0.082	0.539	+0.436	1.379	0.748	0.299	+0.332
II,	1	1.030	0.066	0.738	+0.226	1.078	0.797	0.305	−0.024
	2	1.076	0.046	0.704	+0.326	1.139	0.860	0.326	−0.047
	3	1.091	0.054	0.808	+0.229	1.152	0.776	0.255	+0.121
	4	1.002	0.046	0.793	+0.163	1.019	0.837	0.331	−0.149
	5	1.056	0.054	0.912	+0.090	1.091	0.861	0.318	−0.088
III,	1	1.053	0.059	0.661	+0.333	1.366	0.897	0.398	+0.069
	2	1.068	0.079	0.618	+0.371	1.408	0.931	0.353	+0.124

V. M., female, age 10 years, 3 months; weight 32.4 to 32.0 kilos.

Experiment No.		Intake	Urine	Feces	Balance	Intake	Urine	Feces	Balance
I,	1	0.959	0.098	0.595	+0.266	1.075	0.680	0.296	+0.099
	2	0.985	0.122	0.616	+0.247	1.152	0.767	0.221	+0.164
	3	0.999	0.141	0.588	+0.270	1.193	0.802	0.247	+0.144
II,	1	0.953	0.142	0.709	+0.102	0.931	0.763	0.238	−0.070
	2	0.874	0.105	0.611	+0.158	0.858	0.676	0.214	−0.032
	3	0.889	0.104	0.811	−0.026	0.833	0.634	0.240	−0.041
	4	0.981	0.104	0.736	+0.141	0.959	0.737	0.219	+0.003
	5	0.981	0.090	0.691	+0.200	0.962	0.663	0.224	+0.075

A. M., male, age 6 years, 1 month; weight 17.1 to 17.9 kilos.

Experiment No.		Intake	Urine	Feces	Balance	Intake	Urine	Feces	Balance
I,	1	0.932	0.032	0.669	+0.231	0.999	0.477	0.299	+0.223
	2	0.958	0.031	0.710	+0.217	1.069	0.567	0.298	+0.204
	3	0.964	0.029	0.786	+0.149	1.089	0.620	0.324	+0.145
II,	1	0.876	0.021	0.739	+0.116	0.803	0.555	0.252	−0.004
	2	0.860	0.016	0.694	+0.150	0.803	0.496	0.294	+0.013
	3	0.910	0.015	0.820	+0.075	0.857	0.516	0.281	+0.060
	4	0.897	0.010	0.798	+0.089	0.850	0.526	0.306	+0.022
	5	0.873	0.014	0.767	+0.092	0.815	0.486	0.286	+0.043
III,	1	0.968	0.025	0.642	+0.301	1.106	0.602	0.299	+0.205
	2	0.965	0.025	0.638	+0.302	1.099	0.306	0.344	+0.449

The six children studied by us will be seen to have given somewhat variable results as regards storage of phosphorus on the vegetable-rich diets. In some cases (third series) they stored as much phosphorus from the vegetable-rich as from the milk-rich diets, while in other cases the phosphorus balance like the calcium balance was very unfavorably affected when vegetables were substituted for half of the milk in the diet.

TABLE XIV.

Average Calcium and Phosphorus Balances for the Fourth Series of Experiments, in Grams per Day.

Experiment No.	Calcium.				Phosphorus.			
	Intake.	Output.	Balance.	Balance per kilo.	Intake	Output	Balance.	Balance per kilo.
L. M., female, age 12 years, 9 months; weight 48 kilos.								
	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.
I	1.044	0.605	+0.430	+0.000	1.336	1.088	+0.248	+0.005
II	1.051	0.844	+0.207	+0.004	1.096	1.133	−0.037	−0.001
III	1.060	0.708	+0.352	+0.007	1.387	1.290	+0.097	+0.002
V. M., female, age 10 years, 3 months; weight 32.4 to 32.0 kilos.								
I	0.981	0.720	+0.261	+0.008	1.140	1.004	+0.136	+0.004
II	0.939	0.824	+0.115	+0.004	0.911	0.924	−0.013	±0.000
A. M., male, age 6 years, 1 month; weight 17.1 to 17.9 kilos.								
I	0.951	0.752	+0.199	+0.011	1.052	0.861	+0.191	+0.011
II	0.894	0.789	+0.105	+0.006	0.833	0.806	+0.027	+0.002
III	0.967	0.665	+0.302	+0.017	1.103	0.776	+0.327	+0.018

SUMMARY AND CONCLUSIONS.

The complete balance of intake and output of calcium (and in most cases also of phosphorus) has been determined in four progressive series of experiments including in all 21 children between the ages of 3 and 14 years and covering a total of 417 experimental days in 139 experiments of 3 days each.

On an ordinary mixed diet containing daily 750 gm. of milk and furnishing a total of 0.74 to 1.02 gm. of calcium per day, children 3 to 13 years of age stored 0.15 to 0.62 gm. of calcium per day, the

amount being approximately proportional to the size of the child and averaging 0.01 gm. of calcium per kilo of body weight per day.

When the daily allowance of milk was increased to 1,000 gm. the storage of calcium was increased. The results obtained indicate that optimum storage of calcium is made when the diet contains 1 quart of milk per day for each child. This, with a normal allowance of other foods, will usually mean a daily intake of at least 1 gm. of calcium for the growing child.

Children do not seem to utilize the calcium of vegetables as efficiently as they do that of milk. In the experiments here reported the calcium balances were more variable and always less favorable when vegetables replaced about half of the milk as source of calcium.

In general the conditions influencing the storage of calcium tended to influence that of phosphorus in the same direction; but as all the experiments here described were planned primarily with reference to calcium, the data for phosphorus are not discussed in corresponding detail.

The finding here reported, that children from 3 to 13 years old require an intake of a gram of calcium per day to induce optimum storage of this element has an important bearing upon standards for calcium in family dietaries. It has been found that the average requirement for maintenance is 0.45 gm. of calcium per man per day, and it has been customary to allow a margin of 50 per cent above this actual maintenance requirement thus making an allowance of 0.68 gm. per day as a "dietary standard" for calcium. Since the child of 3 to 13 years, although eating less food than the man, will need more than 0.68 gm. of calcium in his food to support optimum calcium storage and bone and tooth development, it would seem that a higher dietary standard for calcium, perhaps 1 gm. or more per man per day, would be better in all cases in which the group of people to be fed includes any growing children.

In view of the results obtained with different foods as sources of calcium it is desirable also to emphasize the importance of a quart of milk per day for every child, and it would be best to maintain this level of milk intake up to at least the age of 12 to 14 years.

BIBLIOGRAPHY.

1. Sherman, H. C., Mettler, A. J., and Sinclair, J. E., *U. S. Dept. Agric., Office Exp. Station, Bull. 227*, 1910.
2. Sherman, H. C., *J. Biol. Chem.*, 1920, xliv, 21.
3. Sherman, H. C., Chemistry of food and nutrition, New York, 2nd edition, 1918, Chapters IX and X. (See brief bibliography at the end of each chapter.)
4. Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1918, xxxiv, 131.
5. McCollum, E. V., Simmonds, N., and Parsons, H. T., *J. Biol. Chem.*, 1919, xxxviii, 113; and numerous other papers.
6. Hoffström, K. A., *Skand. Arch. Physiol.*, 1910, xxiii, 327.
7. Herbst, O., *Johrb. Kinderh.*, 1912, lxxvi, 40.
8. Herbst, O., *Z. Kinderh.*, 1913, vii, 161.
9. Birk, W., *Monatschr. Kinderh.*, 1908, vii, 450.
10. Birk, W., *Monatschr. Kinderh.*, 1910-11, ix, 595.
11. Cronheim, W., and Müller, E., *Johrb. Kinderh.*, 1903, lvii, 45.
12. Orgler, A., *Monatschr. Kinderh.*, 1911, x, *Orig.*, 373.
13. Schlossmann, A., *Arch. Kinderh.*, 1905, xl, 1.
14. Tobler, L., and Noll, F., *Monatschr. Kinderh.*, 1910-11, ix, 210.
15. Tangl, F., *Arch. ges. Physiol.*, 1904, civ, 453.
16. Bruck, A. W., *Monatschr. Kinderh.*, 1907-08, vi, 570.
17. Cronheim, W., and Müller, E., *Biochem. Z.*, 1908, ix, 76.
18. Rothberg, O., *Jahrb. Kinderh.*, 1907, lxvi, 69.
19. Blauberg, M., *Z. Biol.*, 1900, xl, 1.
20. Blauberg, M., *Z. Biol.*, 1900, xl, 36.
21. Wolff, G., *Jahrb. Kinderh.*, 1912, lxxvi, 180.
22. Niemann, A., *Jahrb. Kinderh.*, 1912, lxxv, 533.
23. Meyer, L. F., *Biochem. Z.*, 1908, xii, 422.
24. McCrudden, F. H., and Fales, H. L., *J. Exp. Med.*, 1912, xv, 450.
25. Klotz, M., *Jahrb. Kinderh.*, 1909-10, lxx, 1.
26. Schabad, J. A., *Arch. Kinderh.*, 1910, liv, 83.
27. Schabad, J. A., *Z. klin. Med.*, 1909, lxvii, 454.
28. Czerny, A., and Keller, A., Des Kindes Ernährung, Ernährungstörungen und Ernährungstherapie, Leipsic and Vienna, 1906, i, 292-298.
29. Porter, W. J., *Am. J. Physiol.*, 1920, lii, 121.
30. Rose, M. S., *J. Biol. Chem.*, 1920, xli, 349.
31. Blatherwick, N. R., and Long, M. L., *J. Biol. Chem.*, 1922, lii, 125.
32. McClugage, H. B., and Mendel, L. B., *J. Biol. Chem.*, 1918, xxxv, 353.

THE INFLUENCE OF POSITION AND OF TEMPERATURE UPON THE REACTION OF ALIPHATIC AMINO NITROGEN WITH NITROUS ACID.

By MAX S. DUNN and CARL L. A. SCHMIDT.

(From the Department of Biochemistry and Pharmacology of the University of California, Berkeley.)

The influence of molecular structure on chemical reactivity is illustrated by a comparison of the time required for the α-amino and the ϵ-amino nitrogen of lysine to react with HNO_2. Van Slyke[1] found that while the α-amino groups of the amino-acids yield their nitrogen completely in 4 to 5 minutes when shaken with HNO_2 a half hour is required for the ϵ-amino group to react quantitatively. The velocity of the reaction is markedly influenced by temperature. At 24°C. the ϵ-amino group of lysine yields its nitrogen in 15 minutes and, according to Sure and Hart,[2] the reaction is completed in 5 minutes by raising the temperature to 37°C. Van Slyke's experiments suggest that the reaction between amino nitrogen and HNO_2 is one of the first order. He found that at 19°C. approximately 70 per cent of the ϵ-amino nitrogen is set free in the first 5 minutes and an additional 25 minutes are required in order that the reaction may be completed. Sure and Hart[2] treated lysine with HNO_2 at 1°C. and measured the gas which was given off after shaking for 5 minutes. They found that the yield of nitrogen corresponded to one-half of the nitrogen content of lysine and from this fact they conclude that low temperatures have a retarding effect on the ϵ-amino and not on the α-amino group of lysine. Without giving due consideration to the time factor Sure and Hart conclude that at 1°C. it is possible to render the ϵ-amino group of lysine entirely inactive.

[1] Van Slyke, D. D., *J. Biol. Chem.*, 1911, ix, 199; 1912, xii, 282.

[2] Sure, B., and Hart, E. B., *J. Biol. Chem.*, 1917, xxxi, 527.

The seemingly sweeping conclusions of Sure and Hart indicate a somewhat paradoxical condition in lysine; *viz.*, that lowering of temperature is without influence on the reactivity of the α-amino group while the same factor serves to prevent the reaction between HNO_2 and the ϵ-amino group. It appears to us that the facts are more nearly in accord with the dynamics of chemical reactions if it be assumed that both reactions are influenced by the factor of temperature. Since, however, the velocity of the reaction between HNO_2 and the α-amino nitrogen is large as compared with that of the ϵ-amino group, a lowering of the temperature, while in reality retarding both reactions, seemingly appears to inhibit the latter reaction entirely. Estimation of the amount of amino nitrogen which is given off when lysine is treated with HNO_2 for a period of 5 minutes is an insufficient criterion on which to base the conclusions which are advanced by Sure and Hart.

It appeared to us that a study of the rates of reactions of aliphatic amino nitrogen when in positions relative to the carboxyl group varying from the alpha to the epsilon affords an excellent opportunity for the correlation of molecular structure and chemical reactivity. Incidentally we have also studied the influence of temperature on the time required for the α- and the ϵ-amino group to react quantitatively. This information is of both practical and theoretical interest. If the conclusions of Sure and Hart with respect to the inactivity of the ϵ-amino group are correct, a basis would be afforded on which a method for the estimation of the α- and the ϵ- amino nitrogen in protein products could be developed. Our data, however, do not bear out the conclusions of these experimenters.

The alanine, β-alanine, and δ-amino-*n*-valeric acid used in the experiments were Kahlbaum products. The lysine picrate was prepared by the Special Chemicals Co., and the δ-amino-*n*-valeric acid was procured from the Chemical Manufactures Department of the University of Illinois. Estimations of amino nitrogen showed that all of the products were of a high degree of purity. Casein was prepared according to the procedure of Van Slyke and Bosworth[3] except that the treatment with $(NH_4)_2C_2O_4$ was omitted. The solutions of amino-acids were prepared so that a given aliquot would yield nearly exact equivalent amounts of

[3] Van Slyke, L. L., and Bosworth, A. W., *J. Biol. Chem.*, 1913, xiv, 203.

nitrogen. In each instance approximately 15.8 mg. of amino nitrogen were contained in each 25 cc. volumetric flask. A volume of 2 cc. was taken for analysis. This contained approximately 1.26 mg. of nitrogen and yielded slightly more than 2 cc. of gas when the reaction was carried to completion. With the exception of lysine each of the 25 cc. amino-acid solutions contained 2 cc. of 0.1 N NaOH. The lysine picrate was brought into solution by the addition of 11 cc. of 0.1 N NaOH and the casein was dissolved with the aid of 100 mg. of Na_2CO_3. All of the amino nitrogen estimations with the exception of those in which the reaction was allowed to proceed below 15°C. were carried out in a room maintained within 0.5°C. of the indicated temperature by means of a thermoregulator, a hot-plate, and an electric fan. The experiments at 8.5°C. and at 4°C. were carried out in an ice chest. The lower temperature was obtained by placing a large cake of ice near the Van Slyke apparatus and by keeping the air constantly in circulation with the aid of an electric fan. A glass door with conveniently located openings permitted the manipulation of the apparatus.

For the purpose of securing comparable results the following technique was followed in estimating amino nitrogen. All solutions and the apparatus were kept at the indicated temperature for some time before carrying out the estimations of nitrogen. For the lower temperatures it was found necessary to keep the glacial acetic acid at a temperature of 12°C. since at temperatures lower than this it freezes. In order to have the reacting mixture at the indicated temperature, acetic acid at 12°C. was allowed to flow into the reaction chamber, $NaNO_2$ was then added, and the mixture was allowed to stand for some time in order that it might come to the desired temperature. Before adding the solution of amino-acid the temperature of the reaction mixture was taken to insure its being at the same temperature as that indicated by the thermometer on the outside of the apparatus. The speed of the motor was regulated so as to give about 300 vibrations per minute to the deaminizing chamber. 0.5 cc. of water was introduced into the chamber to form a protective layer while admitting the amino-acid solution. This prevented the reaction from proceeding to any considerable extent before the entire amount of amino-acid solution was added. The deaminizing chamber was first shaken by hand in order to secure immediate mixing of the solutions and the shaking was then continued by means of the motor.

The estimation of free amino nitrogen in casein at temperatures above 0°C. was carried out according to the procedure outlined above. The following technique was used in carrying out the reaction at 0°C. A

flask containing 100 cc. of 30 per cent $NaNO_2$ solution and 50 cc. of glacial acetic acid was brought to a temperature of $-0.7°C$. by immersion in a vessel which contained a mixture of equal parts of ice and 66 per cent H_2SO_4. At this temperature there was but little evolution of gas. Dry casein was added to the mixture and the flask was occasionally shaken

TABLE I.

The Influence of the Position of the NH_2 Group in Certain Amino-Acids upon the Rate of Deamination with HNO_2 at 23°C.

Time.	Amino nitrogen liberated.									
	Alanine.		β-Alanine.		γ-Amino-n-valeric acid.		δ-Amino-n-valeric acid.		ε-Amino-caproic acid.*	
min.	per cent	K†	per cent	K†	per cent	K†	per cent	K†	per cent	K†
1.0	55	—	57	—	20	—	—	—	—	—
2.0	84	0.46‡	—	—	—	—	36	—	—	—
2.5	96	0.70§	86	0.33	—	—	—	—	—	—
3.0	99	0.85§	90	0.32	58	0.14	—	—	58	—
3.5	97	0.50‡	96	0.41	—	—	51	0.08	—	—
4.0	—	—	95	0.31	—	—	—	—	—	—
4.5	—	—	97	0.32	74	0.14	—	—	—	—
5.0	100	—	97	0.27	78	0.14	82	0.18	—	—
6.0			—	—	—	—	78	0.12	—	—
6.5			—	—	84	0.12	—	—	—	—
7.0			100	—	—	—	—	—	—	—
8.0					92	0.14	—	—	88	0.11
8.5					—	—	88	0.11	—	—
9.0					95	0.15	—	—	—	—
9.5					—	—	—	—	—	—
10.0			100	—	100	—	97	0.17	—	—
11.5					—	—	92	0.09	—	—
13.0					100	—	100	—	—	—
13.5							—	—	97	0.11
15.0							—	—	101	—
17.0							100	—	100	—
18.0									100	—

* The figures given refer to the ε-amino group of lysine picrate.

† K = Constant for the monomolecular reaction.

‡ Based on the first observer's data.

§ Found by the second observer.

during the course of the reaction. At the conclusion of the desired interval of time the contents of the flask were filtered by suction through a hardened filter paper which had previously been moistened with 10 per cent NaCl solution cooled to $-2°C$. The deaminized casein was washed repeatedly with NaCl solution to remove the HNO_2, care being taken that in all of the

procedures the temperature did not rise above 0°C. The product was successively washed with distilled water, absolute alcohol, and anhydrous ether and dried in air at 60°C. The substance so obtained is a pale, yellow amorphous powder which does not darken on exposure to air.

The experimental results are presented in Tables I to IV. A number of independent estimations with each of the amino-acids used were carried out by each of the writers both as a check and as a measure of the variability inherent in the method. The factor of error reaches its greatest magnitude when the time

TABLE II

The Influence of Temperature Upon the Reaction of Alanine with HNO_2.

Time.	Amino nitrogen liberated at.			
	23°C.	15°C.	8 5°C.	4 0°C.
min.	per cent	per cent	per cent	per cent
1.0	55	41		
2.0	84	82		
2.5	96	—		
3.0	99	88	61	
3.5	97	—	—	
4.0	—	98	—	
5.0	100	100	78	
6.0		100	—	
8.0			97	
10.0			100	
12.0			100	82
15.0				92
18.0				100
21.0				100

The average value for K at 23°C. is 0.48; at 15°C., 0.45; at 8.5°C., 0.22; and at 4°C., 0.12.

interval is short, since the maximum percentage of total amino nitrogen is given off during the first interval of time. Towards the end of the experiment the amount of gas which is given off is so small that it is impossible to determine whether the gas which is measured represents a continuation of the reaction or experimental error. Since it was not possible for us to secure an amino-acid containing only an ε-amino group, use was made of lysine. Estimation of the amino nitrogen in this substance includes, at any time, gas which is given off from both the α- and the ε-amino

group. In order to determine the rate of reaction of the "epsilon" group the first interval of time at which an estimation of nitrogen was carried out was so chosen that all of the α-amino nitrogen

TABLE III.

The Influence of Temperature Upon the Reaction of the ϵ-Amino Group of Lysine Picrate with HNO_2.

Time.	Amino nitrogen liberated at.						
	30°C.	26°C.	23°C.	19°C.	15°C.	8.5°C.	4°C.
min.	per cent	per cent	per cent	per cent	per cent	per cent	per cent
2.0	41						
3.0	67		58				
4.0	89		—				
5.0	—		—-		62		
6.0	95	90	—		—		
8.0	100	99	88		—		
10.0	101	100	—	92	77		
11.0		—	—	92	—		
12.0		100	—	—	—		
13.0			97	97	—		
13.5			101	—-	—		
15.0			100	100	—		
17.0			—	100	—		
18.0			100		—		
20.0					95		
25.0					100		
30.0					100	88	
34.0						93	
38.0						100	
40.0						100	
45.0							94
48.0							97
50.0							100
55.0							100

The average value for at K 30°C. is 0.29; at 23°C., 0.11; and at 15°C., 0.05. The data for other temperatures are insufficient for the calculation of the value for K.

was set free. This time was found from the reaction curve of alanine and in applying these data to the "alpha" group of lysine we do not believe that an error of considerable magnitude is introduced by assuming that the rate of reaction of

the α-amino group of lysine is the same as that of alanine. Our results indicate that the rate of reaction of both the α- and the ϵ-amino group is markedly influenced by temperature. The ϵ-amino group of lysine reacts more slowly than the α-amino group and only about 20 per cent of nitrogen gas will be given off by the former when shaken for 5 minutes with HNO_2 at temperatures near 0°C. The curve (Fig. 1) showing the minimum time required for alanine to yield its nitrogen quan-

TABLE IV.

Influence of Temperature Upon the Reaction of Casein with HNO_2.

Time.	Free amino nitrogen liberated at.			
	21°C.	15°C.	0°C.	
min.	per cent	per cent	per cent*	K†
5.0			21	
10.0			37	0.020
15.0			46	0.017
18.0	83		—	
21.0	95		—	
24.0	98		—	
25.0	—	87	—	
27.0	100	—	—	
29.0	—	98	—	
30.0	100	—	75	0.020
34.0		100	—	
38.0		100	—	
40.0			90	0.026
60.0			100	
120.0			100	

* The difference between the amino nitrogen content of the original untreated casein and the amino nitrogen left in the treated product.

† K = Constant for the monomolecular reaction.

titatively at varying temperatures indicates that within the usual range of room temperature the reaction is complete within a period of 4 minutes. Under similar conditions a quantitative yield of ϵ-amino nitrogen is obtained in a half hour. For practical purposes considerable time may be gained by the use of the temperature curve for the epsilon group of lysine. The curve necessarily includes experimental errors, hence as a factor of safety 2 to 5 minutes, depending on the temperature, more than the time

indicated by the curve should be allowed in the estimation of the ε-amino group of lysine. Comparison of our time values with those given by other investigators for several temperatures shows a satisfactory agreement.

The values for K calculated according to the monomolecular formula show a fair agreement, sufficient however to establish the fact that the reaction between aliphatic amino nitrogen and HNO_2 is of the first order. This is to be expected since the amount of HNO_2 present is in such great excess of that used up by the

Fig. 1. Curve showing the effect of temperature upon the time required for the α- and the ε-NH_2 group to react quantitatively with HNO_2.

Point ⊕ taken from the data of Sure and Hart.

reaction that its concentration is practically a constant and the rate of nitrogen set free is dependent only on the concentration of amino nitrogen. The probable values of K at 23°C. for the amino-acids studied are: alanine 0.48, β-alanine 0.33, γ-amino-n-valeric acid 0.14, δ-amino-n-valeric acid 0.13, ε-amino-caproic acid 0.11. In studying reactions which proceed with the speed as indicated by the values for K considerable experimental error cannot be avoided and this is usually magnified when the estimations are carried out by several observers.

The results also show that the time required for the amino group of the aliphatic amino-acids which were studied, to yield its nitrogen quantitatively when treated with HNO_2 varies directly as the distance from the carboxyl group. Increasing the distance of the amino group from the carboxyl group necessitates a longer period of time in order that a quantitative yield of nitrogen may be obtained. Fig. 2 also shows the relationship between the position of the amino group of the amino-acids studied and the values of K at 23°C. calculated upon the assumption that the reaction between amino nitrogen and HNO_2 obeys the monomolecular formula. When casein is treated with HNO_2 it will be noted that the time which is required in order to obtain all of the free

FIG. 2. Curves showing the relation of the position of the amino group (with reference to the COOH group) in certain amino-acids to: (a) the time required to react quantitatively at 23°C. with HNO_2 and; (b) the value of K at 23°C. calculated upon the assumption that the reaction between amino nitrogen and HNO_2 obeys the monomolecular formula.

amino nitrogen is somewhat greater than that necessary for the deamination of the epsilon group of lysine picrate. However, due consideration must be given to the fact that casein in the presence of acetic acid is precipitated from its solution giving a heterogenous mixture. The factor of surface enters. In order to react, the HNO_2 must penetrate the particles of precipitated casein, hence it is to be expected that the reaction time is prolonged. A fairly good agreement between the reaction time of lysine picrate and of casein is obtained by a comparison of the results which were obtained with lysine at 4°C. and with casein at 0°C. The reaction at the low temperature is sufficiently slow so that the factors of surface and penetration are minimized.

SUMMARY.

The rates of deamination of the α- and of the ϵ-amino groups of certain amino-acids are, between 0 and 30°C. markedly influenced by temperature. Contrary to the statement of Sure and Hart, lowering of the temperature was found to decrease the speed of deamination of the α-amino group (of alanine) and not totally to inhibit the complete liberation of nitrogen from the ϵ-amino group of lysine. The influence of the position of the amino group with respect to the carboxyl group in certain other amino-acids upon the time required to yield their nitrogen quantitatively was also studied.

When treated with HNO_2 at ordinary temperatures casein gives off its nitrogen somewhat more slowly than lysine. Reasons for this difference are advanced.

THE WATER-SOLUBLE CONSTITUENTS OF THE ALFALFA PLANT.*

By THOMAS B. OSBORNE, ALFRED J. WAKEMAN,
AND CHARLES S. LEAVENWORTH.

(*From the Laboratory of the Connecticut Agricultural Experiment Station,
New Haven.*)

In a previous paper[1] we described a method whereby relatively large quantities of a chlorophyll-free juice can be obtained from the alfalfa plant within so short a time that autolytic changes presumably are largely avoided. In the present paper we record observations made respecting some of the chemical constituents of this juice.

Recognizing the fact that we are faced by a chemical problem of great complexity, for the present we have refrained from attempting to isolate definite substances from this juice and have directed our efforts to discovering methods whereby fractions containing groups of substances can be obtained which may afford a better starting point for detailed studies than has heretofore been possible.

We have already shown that adding 20 per cent by weight of alcohol to the clear brown freshly expressed chlorophyll-free alfalfa juice precipitates the "colloids" and that these consist chiefly of protein combined with coloring matters, possibly related to the flavones, together with calcium phosphate. This precipitate, which we have called the "colloid precipitate," was equal to about 18 per cent of the solids of the juice of the plants used for this investigation. We have reason to believe that the proportion of the "colloids" varies somewhat with the age of the

[1] Osborne, T. B., Wakeman, A. J., and Leavenworth, C. S., *J. Biol. Chem.*, 1921, xlix, 63.

plant and the conditions of its growth, and for this reason all quantitative data given in this paper apply only to the particular lot of plants examined and, of course, are only approximate.

On long standing the filtrate from the "colloid precipitate" gradually yields a small deposit which, in one case, was equal to about 1.3 per cent of the solids of the juice. This precipitate, which contained about 18 per cent of ash and 11.5 per cent of nitrogen, consisted largely of protein and calcium phosphate, together with "flavone-like" substances, and behaved towards acids and alkalies as does the "colloid precipitate." This appears to be a residue of the latter which was not immediately precipitated by adding 20 per cent of alcohol to the freshly expressed juice. The filtrate from this small spontaneous separation was perfectly clear, bright red in color and free from all colloids, as shown by the absence of any Tyndall effect when exposed to a beam of sunlight. The substances in this solution are, therefore, to be regarded as in true solution.

When this solution was made neutral to litmus a precipitate, consisting chiefly of calcium phosphate and protein, separated. This, when washed with 20 per cent and stronger alcohol and dried at 105°, was equal to 3.4 per cent of the solids of the press-juice. It contained 4.9 per cent of nitrogen and 63.6 per cent of ash. The $N \times 6.25$ was equal to 31 per cent of protein. If all of the nitrogen belongs to protein this, together with the ash, would account for over 94 per cent of this precipitate.

When the alcohol content of the filtrate from the "colloid precipitate" is raised to 53 per cent by weight by adding an equal volume of 93 per cent alcohol a second precipitate, No. II, is produced, which in the experiment to be described was equal to about 14 per cent of the solids of the fresh alfalfa juice used for the following experiments.

Alfalfa plants from a late cutting (Lot 49) made on Sept. 7, 1921, when the plants were just beginning to blossom, were ground as described in our previous paper,[1] and pressed in the hydraulic press. The first portion of the juice, which was green, was collected separately from the later portion which was brown. The press-cake was ground up with the green juice and pressed again, whereby all of the chlorophyll was retained by the cake. The united brown press-juices measured 4 liters. The press-cakes were washed three times by grinding each time with 4 liters of distilled water and pressing, the final washings being nearly free from color. The results of this procedure are given in the following table.

Alfalfa taken = 7,752 gm. fresh plants, Lot 49.

	Dry solids.*	Nitrogen.	Ash.
	gm.	gm.	gm
Taken.........................	1,863	74.22	168.1
Press-juice.......................	469 60	23 50	79 39
1st wash..........................	139 20	5 05	25 60
2nd "	53 34	1 59	9 00
3rd "	23 85	0 76	2 62
Residue...........................	1,105 00	42 72	47 72
Total...........................	1,790.99	73 62	164 33

* All weights of solids were determined by drying for 24 hours or more at 105° to nearly constant weight. It was practically impossible to obtain a really constant weight for most of the fractions obtained, presumably because these are syrups which retain water with great tenacity. The weights given must therefore be regarded as approximate, but we believe they are sufficiently accurate to serve all the purposes for which they can be reasonably employed in interpreting the results of such a preliminary investigation as is described in this paper. In all cases the weights given are corrected for samples previously taken for analysis.

We thus found that of this lot of alfalfa 36.9 per cent of the dry solids, 41.6 per cent of the nitrogen, and 69.3 per cent of the inorganic matter were soluble in water.

About 5 hours after the alfalfa plants had been cut 1,344 cc. of 93 per cent alcohol were added to the 4 liters of brown press-juice, making the alcohol content of the solution about 20.5 per cent by weight. The voluminous precipitate thereby produced was at once collected on two large folded filters and allowed to drain over night. The next morning an equal volume of 93 per cent alcohol was added to the filtrate making its alcoholic content about 53 per cent. This precipitate was filtered out. The precipitate produced by 20 per cent alcohol, which contained the "colloid" constituents of the juice was very voluminous and retained a large amount of the solution from which it had separated, its total solids being equal to 144.57 gm. When washed twice with 20 per cent alcohol and then with stronger alcohol and ether the residue weighed 87.3 gm., equal to 18.6 per cent of the solids of the alfalfa juice. The second alcohol precipitate, which contained 63.31 gm. of solids, was washed with 53 per cent and then with stronger alcohol and ether, 56.4 gm. remaining undissolved, while 6.9 gm. of soluble matter were removed.

The three successive washings of the press-cakes contained 218.4 gm. of solids. If the 1,863 gm. of dry alfalfa contained the "colloids" in the

proportion which the solids of the press-juice bear to those of the washings of the cake the total "colloid" would be 128 gm. or 6.87 per cent of the dry solids of the plant.

Since 57.3 gm. of solids were removed by washing the "colloid precipitate" the 56.4 gm. of the second alcohol precipitate should be greater by the proportion of this 57.3 gm. of solids which would have been precipitated by 53 per cent alcohol if this had been included in the filtrate. Thus 57.3 gm. + 56.4 gm. Precipitate II + 6.5 gm. in washings of Precipitate II + 256.8 gm. in filtrate from Precipitate II = 377.0 gm., of which 57.3 gm. equals 15.2 per cent. This proportion of the 57.3 gm. retained by the colloid precipitate is 8.7 gm., thus making the total corrected amount of Precipitate II = 65.1 gm. or about 13.8 per cent of the solids of the press-juice. The total solids of the press-juice together with those of the water washings were 686 gm., 13.8 per cent of which is 94.7 or 5.1 per cent of the dry alfalfa.

Summarizing the preceding results on the 4 liters of the un-diluted press-juice (see p. 412) of the fresh alfalfa plants and including determinations of nitrogen and ash we have the following data.

4 liters of the alfalfa juice (Lot 49) contained:

	Solids.	Nitrogen.	Inorganic.
	gm.	*gm.*	*gm.*
"Colloid precipitate"...............	87.3	9.61	11.59
Precipitate II....................	65.1	1.86	32.22
Filtrate from Precipitate II.......	317.2*	12.05	35.58*
Total............................	469.6	23.52	79.39

* Calculated by difference. Directly determined, 312.3 gm. of solids.

We thus find that 40.9 per cent of the nitrogen in the alfalfa juice is precipitated by the addition of 20.5 per cent of alcohol. We have shown in our previous paper[1] that this belongs almost, if not entirely to protein, which, in combination with substances which appear to be similar to flavones, is present in colloidal solution. Precipitate II contains 8 per cent of the original nitrogen, most of which we believe from our examination of this precipitate is likewise protein, and probably largely consists of a small residue of the "colloid" which was not completely pre-cipitated by 20.5 per cent alcohol. Nearly one-half of the nitro-gen of the press-juice, therefore, belongs to protein which is almost

completely removed by adding 53 per cent by weight of alcohol. The alfalfa juice can be thus readily freed from practically all its protein within a very short time after the growing plants are cut and the non-protein nitrogenous constituents obtained in an alcoholic solution so strong that enzymatic, or bacterial changes, will probably not occur. Furthermore, by obtaining the press-juice in the way described substances insoluble in water, but soluble in alcohol, such as chlorophyll, fat, waxes, and phosphatides, do not interfere with a subsequent study of the water-soluble nitrogenous constituents of the plant. Such data as we have obtained respecting these will be given after we have discussed the distribution of the inorganic constituents among the three fractions into which the press-juice has been divided.

Distribution of the Inorganic Constituents of the Alfalfa Juice.

Analysis of the ashes of the three fractions just described, calculated on the basis of 4 liters of the alfalfa juice, gave the following results:

	"Colloid precipitate."	Precipitate II.	Filtrate.	Total.
	gm.	*gm.*	*gm.*	*gm.*
Ca.	4.34	8.32	0.65	13.31
Mg.	0.14	0.53	2.01	2.68
Na.	0.15	0.00	0.85	1.00
K.	0.02	4.08	15.95	20.05
Fe.	0.06	Trace.	0.00	0.06
PO_4.	3.84	7.68	1.99	13.51
SO_4.	0.59	10.51	2.23	13.33
SiO_3.	0.14	0.04	0.16	0.34
CO_3.	2.05	1.48	9.84	13.37
Cl.	0.00	0.00	2.01	2.01
Total.	11.33	32.64	35.69	79.66

The close agreement between the sum of these constituents and the 79.39 gm. calculated from direct determinations of the ash of the solids of the original juice indicates that these analyses fairly represent their true composition.

The proportion of each of the above bases and acids precipitated by 53 per cent alcohol is shown in the following tabulation.

	per cent
Ca	95.1
Mg	25.0
Na	15.0
K	20.4
Fe	100.0
PO₄	85.3
SO₄	83.3
SiO₂	50.0
CO₃	26.4
Cl	None.
Total ash	55.1

These figures show that more than one-half of the inorganic constituents are removed by adding 53 per cent of alcohol and that only a small part of the calcium, phosphoric acid, or sulfuric acid, remains in solution. In so far as carbonic acid represents salts of organic acids it appears that about three-fourths of these are not precipitated. This, however, is not an accurate measure of such salts, because the magnesium salts would leave a corresponding quantity of MgO in the ash. Assuming that all of the Mg was thus combined the proportion of CO_3 corresponding thereto plus that found in the ash is only 20 gm. or about 5 per cent of the organic solids of the press-juice.

The PO_4 is nearly all present as inorganic phosphate as shown by the following experiment.

The precipitate produced by making 100 cc. of the filtrate from the "colloid precipitate" alkaline with NaOH was dissolved in dilute nitric acid, precipitated with molybdic solution, and PO_4 determined in the usual way to be 0.1028 gm.

The filtrate from the precipitate produced by neutralizing the original solution gave no turbidity on adding a few drops of $CaCl_2$. This was then evaporated to dryness, ignited, and only 0.0075 gm. of PO_4 found. Whether this latter really represents inorganic phosphorus or calcium phosphate dissolved in the solution of nitrogenous substances with which it is associated has not been determined, but in any event it is clear that at least 95 per cent of the PO_4 is present as PO_4 ions.

The same is true of the SO_4 ions, direct determinations made under different conditions agreeing closely with those made on the ash. It is interesting to note that practically all of the iron is present in the "colloid" precipitate which, when washed with dilute and strong alcohol and dried over H_2SO_4, reacts with both ferro- and ferricyanide.

The magnesium, which is chiefly in the filtrate, is all present in inorganic form as direct determinations made under conditions which should preclude hydrolysis of organic compounds gave the same result as those made on the ash.

Thus to 100 cc. of the filtrate from the ' colloid precipitate" $(NH_4)_2C_2O_4$ was added and the calcium oxalate removed by centrifuging. To the clear solution NH_4OH and Na_2HPO_4 were added and after stirring and standing over night the precipitate was removed by centrifuging, dissolved in dilute HCl, and reprecipitated as $MgNH_4PO_4$, equal to 0.0429 gm. of Mg. This agrees exactly with the Mg found in the ash from 100 cc. of the same solution.

The Nitrogen of the Alfalfa Juice.

Although the nitrogenous constituents of the alfalfa juice undergo continuous changes during the growth of the plant and can therefore not be dealt with from a quantitative, or even a qualitative standpoint, with any high degree of accuracy, nevertheless, we found that it is possible to obtain analytical results which should contribute ultimately to a better knowledge of these substances than we now have.

The agricultural chemist formerly regarded the water-soluble non-protein nitrogen as belonging chiefly to amides and it was customary in stating the results of analyses of green fodders to designate this as "amide nitrogen." Later, when it became known that amino-acids were the chief products of protein hydrolysis it seems to have become customary to believe that a large part of this nitrogen belongs to amino-acids, or their peptides, although satisfactory chemical evidence of this, so far as we can find, has never been presented.[2] The filtrate from Precipitate II, which contains one-half of the nitrogen of the alfalfa juice presents an opportunity for attacking this problem under more favorable conditions than those heretofore encountered, because practically all of the protein and other colloidal substances, as well as a considerable part of the inorganic constituents have been removed. In the following pages we record the results of preliminary studies of the various types of nitrogen in this solution.

[2] For a discussion of the literature see Hart, E. B., Humphrey, G. C., and Morrison, F. B., *J. Biol. Chem.*, 1912–13, xiii, 133.

The 4 liters of juice of the alfalfa plants (Lot 49), a part of which was used for the analyses of the inorganic constituents just described, contained 23.5 gm. of nitrogen. Of this the colloid precipitate contained 9.61 gm. and Precipitate II 1.90 gm., in all 49.5 per cent of the total nitrogen of the juice. As already stated, nearly all of this nitrogen appears to belong to protein, although as yet our evidence is not entirely convincing that a small part may not belong to non-protein substances insoluble in 53 per cent alcohol. The 50.3 per cent of the nitrogen in the filtrate from Precipitate II is equal to 16.1 per cent of the total nitrogen in this lot of alfalfa.

In 100 cc. of this filtrate, which contained 0.120 gm. of total nitrogen and 53 per cent of alcohol, *ammonia* was determined by adding about 300 cc. of water and a slight excess of MgO and then distilling to a small volume. Ammonia equal to 5.75 per cent of the total nitrogen was found in the distillate. This is equivalent to 2.51 per cent of the total nitrogen in the alfalfa juice, which contained 31.7 per cent of the total nitrogen in the alfalfa. Another determination was made by evaporating 150 cc. of this filtrate on the steam bath, dissolving the residue in 400 cc. of water, and distilling with MgO as before. In this case 5.6 per cent of the nitrogen was found as ammonia.

Unless this solution contains extremely unstable nitrogenous compounds it thus appears that practically all of the ammonia was present as ammonium salts.

When the concentrated solutions, from which the ammonia had been removed, were centrifuged, a relatively large orange-yellow deposit was obtained. When this was washed with water, treated with strong HCl, and the solution shaken with butyl alcohol the aqueous layer was light yellow and the butyl alcohol layer deep ruby-red.

The MgO had, therefore, precipitated a considerable quantity of the coloring matter.

Basic nitrogen was determined by concentrating the filtrate and washings from the MgO precipitate to 100 cc., adding 5 per cent by weight of H_2SO_4 and then a 20 per cent solution of phosphotungstic acid in 5 per cent H_2SO_4 as long as a precipitate formed. This required about 30 cc. of this reagent. The precipitate was washed with 2.5 per cent phosphotungstic acid in 5 per cent H_2SO_4 solution and the nitrogen determined in it to be equal to 44.4 per cent of the total nitrogen in the filtrate from Precipitate II.

Two other portions of 150 cc. each of the filtrate from Precipitate II, containing 0.1800 gm. of N, were acidified with HCl, evaporated on the steam bath, the residue was dissolved in 75 cc. of 20 per cent HCl and boiled under a reflux for 14 hours. After evaporating the solutions to dryness the residues were dissolved in 400 cc. of water, and distilled with an excess of MgO.

The ammonia found in the distillate, obtained by boiling the solutions to small volume, in each case was equal to 17.0 per cent of the total nitrogen in the filtrate from Precipitate II. or to about three times as much as before hydrolysis.

After distilling off the ammonia the residue, which was removed by centrifuging, was black and contained a relatively large proportion of humin, presumably derived chiefly from carbohydrates. Nitrogen equal to 11.2 per cent of the nitrogen in the filtrate from Precipitate II was found in the "humin" from one of these determinations. The "humin" from the other determination was treated with strong HCl and shaken with butyl alcohol. The aqueous layer was only slightly colored, but the butyl alcohol layer after centrifuging had a very deep ruby-red color, while the humin formed a coherent mass at the bottom of the butyl alcohol. This layer was washed twice with water and nitrogen determined in both layers. The aqueous solution contained nitrogen equal to 5.4 per cent of the total nitrogen in the filtrate from Precipitate II; the butyl alcohol layer, including the humin, contained 7.8 per cent.

The distribution of nitrogen was similarly determined in the juice of another lot (No. 54) of alfalfa with results as stated below together with those just described for Lot 49.

Since the 53 per cent alcohol solutions used for these determinations had been kept for several months similar analyses were made on a corresponding solution which had been kept for only 3 or 4 days, No. 57.

The distribution of nitrogen both before and after hydrolysis is given in the table below in percentage of the nitrogen in the filtrate from Precipitate II.

	No. 49	No. 54	No. 57
Before hydrolysis.			
Ammonia nitrogen	5.7	3.0	4.5
"Basic nitrogen"	44.4	38.7	51.3
Nitrogen in the MgO precipitate	1.3		1.4
Free amino nitrogen	29.2	25.2	23.8
After hydrolysis.			
Ammonia nitrogen	17.0	16.9	14.2
"Basic nitrogen"	20.4	25.9	19.0
Nitrogen in the MgO precipitate	11.2	6.9	16.4
Free amino nitrogen	41.7	46.3	38.3

The fact that a similar small percentage of the nitrogen was obtained as ammonia from the freshly made unhydrolyzed solution from Lot 57 as from those from Lots 49 and 54 which had been kept 8 months indicates that little, if any, change takes place in the nitrogenous constituents when kept in the 53 per cent alcohol solution.

Although these results were obtained with different lots of alfalfa by methods devised for dealing with the products of hydrolysis of proteins the agreement between the analysis is even closer than might be expected. It is evident that the proportion of ammonia nitrogen is greatly increased by hydrolysis while that of the "basic nitrogen" is greatly decreased. Before hydrolysis about one-fourth of the nitrogen reacts with nitrous acid as does free amino nitrogen, after hydrolysis about 40 per cent reacts in this way.

The basic substances from the *unhydrolyzed* filtrate from Precipitate II were examined for arginine, histidine, and lysine by Kossel's method. Although about 8 per cent of the nitrogen in the filtrate from Precipitate II was precipitated by silver nitrate and baryta from the solution obtained by decomposing the phosphotungstic acid precipitate no evidence of the presence of arginine or lysine was obtained. The color reaction for histidine with diazobenzenesulfonic acid as well as the precipitate which formed on adding $HgSO_4$ indicated the presence of this aminoacid, but both of these reactions are given by other nitrogenous substances and consequently cannot be accepted as proof that histidine was actually present.

About 20 liters of the filtrate from Lot 54 were concentrated *in vacuo* at a low temperature to 1,500 cc. This concentrated solution contained 352 gm. of solids, 22.56 gm. of N, and yielded 53.33 gm. of ash, containing 25.6 per cent of CO_3.

Part of this solution, containing 7.9 gm. of solids and 0.509 gm. of N, was diluted to 300 cc. and 5 per cent H_2SO_4 added. Phosphotungstic acid (20 per cent solution in 5 per cent H_2SO_4) was added as long as a precipitate was produced. This was washed with dilute phosphotungstic acid solution and decomposed at room temperature by an excess of baryta. The filtrate and washings from the insoluble barium salts were treated with CO_2, concentrated to small volume, the $BaCO_3$ was washed with water, and the filtrate and washings were made up to 250 cc. Analyses of aliquots of this solution showed it to contain

		N in filtrate from Precipitate II.
	gm.	*per cent*
Total basic nitrogen............................	0 1150	22.6
Amino nitrogen................................	0 0155	3.0
N in second basic N precipitate................	0 0920	18 1

The total basic nitrogen is equal to only 22.6 per cent of the total nitrogen contained in the filtrate from Precipitate II as against 38.7 per cent found in the phosphotungstic acid precipitate in another portion of this same solution and 44.4 per cent in the corresponding precipitate from Lot 49, previously described. These facts indicate that in decomposing the phosphotungstates with baryta nearly one-half of the basic nitrogen was removed with the baryta precipitates. As this possibility was not foreseen nitrogen was not determined in these precipitates.

The free amino nitrogen is equal to only 13.5 per cent of the total basic nitrogen in the filtrate from the barium salts, a proportion much smaller than should have been found if this basic nitrogen were yielded by arginine, histidine, or lysine.

That arginine, or other substances which yield ammonia on boiling with alkalies, was absent is shown by the fact that when 25 cc. of the 250 cc. of the above solution were diluted with about 400 cc. of 1 per cent NaOH solution and distilled to small volume no ammonia was found in the distillate.

When another portion of 50 cc. of this solution was precipitated by phosphotungstic acid in the usual way 80 per cent of its basic nitrogen was found in the precipitate. In view of the known solubility of most phosphotungstates it appears that practically all of the nitrogen in the 250 cc. of this solution belongs to strongly basic substances.

The remaining 115 cc. of this solution was boiled with an equal volume of concentrated HCl for 7 hours and found to contain 0.0820 gm. of basic nitrogen and 0.0380 gm. of free amino nitrogen, calculated to the original 250 cc. of the solution analyzed. The basic nitrogen thus found after hydrolysis was equal to 71 per cent of the total basic nitrogen as against 80 per cent similarly found before hydrolysis, while the free amino nitrogen had increased from 13.5 to 33.0 per cent of the basic nitrogen. It will require a repetition of this experiment on a scale sufficiently large to permit of the actual isolation of the basic substances before these results can be interpreted.

In another portion of the concentrated filtrate from Precipitate II (Lot 54), containing 0.3008 gm. of N, ammonia was determined by distilling with MgO to be equal to 2.8 per cent of its total nitrogen. After

removing the MgO precipitate and washing this with water, the solution and washings were concentrated *in vacuo* to 200 cc. Of this, 170 cc. were acidified with 5 per cent of H_2SO_4 and precipitated with phosphotungstic acid as described in the preceding experiments. The washed phosphotungstates were decomposed with baryta, the excess of baryta was removed as $BaCO_3$, and the filtrate concentrated. This solution, which reacted strongly alkaline to litmus, was acidified with nitric acid and then treated alternately with silver nitrate and baryta until an excess of silver had been added. The silver precipitate was then decomposed with H_2S in dilute H_2SO_4 solution and the filtrate and washings from the Ag_2S were concentrated to 100 cc. and nitrogen was determined in 10 cc. to be equal to 0.0230 gm. in the whole, or to only 7.7 per cent of the nitrogen in the filtrate from precipitate II. As we have already given evidence of the absence of arginine in the phosphotungstic acid precipitate this nitrogen may represent histidine. Qualitative tests for histidine with diazobenzenesulfonic acid and also with $HgSO_4$ solution gave positive results, but these cannot be accepted as final until this amino-acid is actually isolated.

The filtrate from the silver nitrate-baryta precipitate was freed from silver and baryta by H_2SO_4 and H_2S, made up to 200 cc., and 5 per cent H_2SO_4 added. Phosphotungstic acid was added to this solution as long as a precipitate was produced which was then washed and decomposed with baryta. After removing the excess of barium with carbonic acid the filtrate was concentrated and treated with picric acid under the conditions employed by Kossel for the precipitation of lysine. A relatively large precipitate separated which, when recrystallized, separated in needles somewhat resembling lysine picrate, but which proved to be potassium picrate.[3] The mother liquor when concentrated to a very small volume and allowed to evaporate to dryness left a residue which crystallized in plates. These crystals were insoluble in ether, sparingly soluble in absolute alcohol, but readily soluble in 50 per cent alcohol. No lysine picrate could be isolated.

It is evident that most of the basic nitrogen obtained from the filtrate from Precipitate II belongs to nitrogenous substances quite different from those yielded by protein hydrolysis.

The Coloring Matters of Alfalfa Juice.

The freshly expressed juice of the alfalfa plant is strongly colored and its color for the most part remains in solution after

[3] Potassium phosphotungstate is so insoluble that in working with solutions rich in potassium salts a relatively large proportion may be precipitated with the nitrogenous bases. Since potassium picrate can be easily mistaken for lysine picrate there is danger of a serious explosion if its melting point is taken.

removing the "colloid precipitate" as well as that produced by the addition of 53 per cent alcohol. The concentrated filtrate from this latter precipitate when viewed by reflected light is black, but by transmitted light a clear ruby-red.

Much, if not all, of this color is due to the presence of substances which may belong to the group of flavones, but we want it to be distinctly understood, that as yet we have *no chemical* evidence sufficient to support this view. A part of this color can be removed from the concentrated filtrate by shaking with isoamyl alcohol and the aqueous layer is thus rendered much lighter in color, hence we conclude that a part of the color is in the free state.

After acidifying the aqueous layer and again shaking with isoamyl alcohol a somewhat larger quantity is removed, indicating that acids liberate this coloring matter from salt-like combination.

On boiling the acidified aqueous layer under a reflux condenser a highly hydrated, light brownish product separates which somewhat resembles coagulated protein, but is nearly all soluble in absolute ethyl alcohol with a characteristic deep brown color. In how far this is chemically similar to the products extracted by isoamyl alcohol remains for further investigation.

After removing this insoluble product the aqueous solution is darker than before boiling with acids and contains a relatively large amount of substance soluble in isoamyl alcohol with the same characteristic color as that of the fractions previously extracted by this reagent. In view of the fact that flavones are usually obtained from vegetable sources as glucosides it is probable that the last fraction of the coloring matter was liberated by hydrolysis of some complex of still unknown nature. We have not as yet been able to determine the relative proportions of these four fractions of the coloring matter because none have been obtained in a state of probable purity, nor were any of them free from nitrogen. For this reason we are not prepared to state the proportion in which these highly colored substances occur in the alfalfa juice, but the data which follow show that they unquestionably constitute an important part of the solid matters of the alfalfa juice, and those given in our former paper[1] that they likewise form a considerable part of the insoluble solids of the plant

For the most part these flavone-like substances are combined with the protein and other constituents of the plant from which they are set free by hydrolysis.

That these are widely distributed constituents of vegetable cells and have a physiological importance hitherto unappreciated is indicated by the fact that when colorless yeast cells are hydrolyzed by boiling with HCl and the hydrolysate is shaken with isoamyl alcohol, the latter separates from the aqueous solution with a deep red color not to be distinguished by the eye from that similarly obtained from the alfalfa. This latter fact also indicates that these colored substances are not especially concerned in photosynthetic processes for such do not occur in the yeast cell. These substances deserve further careful study.

The experimental work on which the above statements are founded is described in the following pages.

When about 1.3 per cent of HCl is added to the filtrate from the colloid precipitate a small precipitate is produced which contains both protein and highly colored substances soluble in absolute alcohol, the behavior of which suggests flavone derivatives. This precipitate, which is equal to about 3.5 per cent of the solids of the juice contains nitrogen equivalent to one-third of its weight of protein. The coloring matter could be largely extracted from the precipitate by alcohol containing a very little HCl which suggests that a part of it may be united with the protein in salt-like combination. It is also possible that some of this colored substance was present in the juice as a salt of some inorganic base and that when liberated forms an insoluble compound with the protein.

When the filtrate from the above precipitate is heated on the steam bath for a few hours a voluminous, buff-colored precipitate gradually separates in clumps and skins on the surface of the solution. When this product is removed by centrifuging it forms a dense, dark brown deposit which is almost completely soluble in absolute alcohol. If the solution is boiled with acid stronger than 1.3 per cent, e.g. 5 per cent, the amount insoluble in absolute alcohol is greater. This insoluble part consists of a dense black substance resembling humus, which may have originated from carbohydrates.

The solution of the coloring matter in absolute alcohol is very deep ruby-red. On evaporation it leaves a deep brown residue, readily soluble again in absolute alcohol, or in dilute aqueous NaOH solution. This latter solution appears black by reflected light, or deep yellow by light transmitted through thin layers, and is canary-yellow when highly diluted. The alkaline solution yields a chocolate brown flocculent precipitate when neutralized. This precipitate is slightly soluble in water and, when freed from soluble salts, readily passes into a colloidal solution.

No preparation of this product of hydrolysis has yet been obtained free from nitrogen and no method has yet been discovered whereby it can be purified. For this reason we are not yet prepared to venture an opinion respecting its chemical nature, but as we are already in possession of relatively large quantities of material we hope to be able to obtain some evidence of value in the near future.

This substance, which is obviously a product of hydrolysis, of some complex present in the alfalfa, forms about 3 per cent of the solids of the juice. The total amount of the flavone-like substance which is precipitated by acid together with that which subsequently gradually separates on heating is equal to about 5 per cent of the solids of the juice. The filtrate from these precipitates has a deep ruby-red color and still contains much coloring matter.

By repeatedly shaking this solution with isoamyl alcohol a large part of the color is extracted, but at the same time a relatively large amount of hydrochlorides of nitrogenous substances pass into the amyl alcohol. Thus by thrice extracting with isoamyl alcohol and separately evaporating the three successive extracts to small volume *in vacuo* and then repeatedly shaking each with water it was found that the amyl alcohol extracts contained respectively, 2.68, 1.92, and 3.47 per cent of nitrogen, while at the same time a part of the coloring matter had again passed into water used for washing them. Although the nitrogen content of the solids originally extracted by amyl alcohol was reduced to less than one-third, by washing with water it was not practicable to effect a satisfactory separation of its nitrogenous constituents in this way.

The fact that the three fractions above mentioned were together equal to over 10 per cent of the solids of the juice indicates that the colored products of hydrolysis of the water-soluble constituents of the alfalfa juice, even after removing those which separate as solids on boiling with acid, are present in relatively large amount. In view of the fact that flavones unite with both acids or alkalies to form products soluble in water, it is to be expected that a sharp separation of these from hydrochlorides of amino-acids and probably other nitrogenous bases could not be effected in the above described manner. Although we have tried many experiments to make this separation more effective none has, as yet, proved successful.

Shaking with amyl alcohol does, however, effect a good separation from the inorganic constituents of the juice, and we believe also from a large part of the non-nitrogenous substances. We hope, therefore, that ultimately this method may prove helpful in obtaining fractions from which not only coloring substances, but also some of the nitrogenous constituents can be obtained in definite form. Other immiscible solvents such as ether, benzene, toluene, petroleum ether, ethyl acetate, or chloroform remove almost nothing from this solution. Butyl alcohol on the other hand extracts the coloring matter readily and, at the same time, also a large part of the nitrogenous substances. Further experience with this solvent may lead to better results than did isoamyl alcohol, but with either solvent some way must be found to separate the coloring matters from the nitrogenous hydrochlorides.

In one experiment the filtrate from the precipitate produced by 53 per cent alcohol, containing 300 gm. of solids, was concentrated to 1 liter and shaken with amyl alcohol. When the extract was washed with water only 6.8 gm. of solids, containing 4.5 per cent of nitrogen and 0.4 per cent of ash, were found to have been extracted. This shows that only a small part of the flavone-like substances, which are soluble in amyl alcohol, was present in the free state. By acidifying the water-soluble part (1,225 cc.) with 2.5 per cent of hydrochloric acid and again shaking with amyl alcohol and washing the extract with water after it had been concentrated *in vacuo*, 7.1 gm. of solids containing 2 per cent of nitrogen remained in the amyl alcohol, indicating that a further part of the coloring matter was liberated from salt-like combination by the acid. When the water-soluble part was concentrated *in vacuo* and boiled for $5\frac{1}{2}$ hours at such an acid reaction as was caused by the addition of 2.5 per cent of HCl, 10.5 gm. of solids, containing 3.0 per cent of nitrogen and 0.3 per cent of ash, separated as a voluminous precipitate. More than one-half of this fraction was soluble in absolute ethyl alcohol, the solution being almost black in color. On again shaking the filtrate with amyl alcohol, and washing the extract with water, 10.5 gm. of solids, containing 1.9 per cent of nitrogen, were obtained, indicating that hydrolysis liberated a further quantity of the coloring matter.

When this solution was concentrated *in vacuo* and more HCl added, equal to 7.2 per cent, 2.0 gm. more of solids were obtained from the amyl alcohol extract, after this had been washed with water. The aqueous solution was then boiled for four successive periods of 7 hours, and filtered after each. The insoluble matter which separated was washed with water and alcohol. The black humus-like residue weighed 12.4 gm. and contained 4.3 per cent of nitrogen. This fraction which was insoluble in alcohol was probably chiefly humin derived from carbohydrates. The dark-colored alcoholic washings of this humin weighed 2.87 gm. and contained 4.1 per cent of

nitrogen, showing that a little more of the coloring matter had separated on further hydrolysis.

Although 73.3 gm. of HCl had been added up to this point nevertheless when the filtrate from the insoluble hydrolysis products was concentrated to a syrup the acidity of the distillate, as estimated by titration of aliquots, was equal to only 9.2 gm. of HCl and the acidity of this was the same to methyl red as to phenolphthalein.

From this we conclude the solids in this filtrate include a relatively large proportion of strongly basic substances and that notable quantities of volatile organic acids are absent.

The solution was then extracted eleven successive times with 200 cc. of butyl alcohol and the united extracts were concentrated and washed ten times with water. There then remained in the butyl alcohol 19.44 gm. of solids containing 6.0 per cent of nitrogen. The first two water washings were returned to the main solution and the remaining eight concentrated and found to contain 6.2 gm., containing 7.9 per cent, of nitrogen.

The main aqueous solution was then concentrated and HCl added in quantity sufficient, with that already present, to make 15 per cent in all. This solution was then shaken twice with amyl alcohol and each of the extracts was washed with water and solids equal to 9.61 gm., containing 6.0 per cent of nitrogen, were found to have been extracted. Only 1.5 gm. of solids, containing 8.2 per cent of nitrogen, were removed by washing with water, indicating that the greater part of the substance extracted by amyl alcohol is much more readily soluble therein than in water.

The main solution was next extracted 31 times using 400 cc. of normal butyl alcohol each time. Since normal butyl alcohol dissolves a notable quantity of water the volume of the aqueous solution was maintained at about 1,000 cc. by additions of water. The united butyl alcohol extracts were concentrated in vacuo to about 300 cc., the solids which separated were removed by centrifuging and washed with butyl alcohol. These weighed 8.6 gm. and contained 0.608 gm. of N and 6.08 gm. of ash. The fixed ash was equal to 71 per cent and the nitrogen to 24.2 per cent of the volatile part. Since distillation with MgO converted over 93 per cent of this nitrogen into ammonia it is evident that this fraction consisted of inorganic and ammonium salts. The butyl alcohol solution when made up to 500 cc. contained 103.5 gm. of solids, 6.23 gm. of N, and 2.51 gm. of ash, or one-third of both the solids and the nitrogen of the filtrate from the 53 per cent alcohol, but only 5.5 per cent of the ash. This butyl alcohol solution was next washed 26 times with water, 50 cc. being used each time. There were thus removed 82.1 gm. of solids, 6.9 gm. of N, and 2.3 gm. of ash. The butyl alcohol solution was made up to 500 cc. When 5 cc. of this were evaporated in the steam bath and the residue was dried over night at 105° it weighed 0.1535 gm. This, however, on further heating gradually lost weight so that after 24 hours it weighed 0.0933 gm. and after 10 days continuously at 105° weighed only 0.0787 gm. Since we do not know the nature

of the solids in this solution it is at present impossible to determine the cause of this continued loss of weight, nor the amount of solids in this solution or their percentage content of nitrogen. If, as is possible, the butyl alcohol solution contained hydrochlorides of amino-acids these might be converted into butyl esters which would probably be slowly volatile at 105°.

Although the amount of total solids in this solution is thus uncertain it is evident that even taking the highest figure, 15.35 gm., by far the greater part of the solids extracted by normal butyl alcohol again passed into aqueous solution when the butyl alcohol extracts were repeatedly shaken with water.

The main solution which had been extracted 31 times with butyl alcohol as just described, contained 98.5 gm. of solids, 5.14 gm. of N, and 38.1 gm. of ash.

Thus about one-third of the solids, one-fourth of the nitrogen, and more than four-fifths of the inorganic constituents still remained in solution after these repeated extractions, first with isoamyl alcohol and then with butyl alcohol.

Of the total nitrogen left in this solution 1.347 gm. were obtained as ammonia on distilling with MgO and 2.13 as amino nitrogen by the Van Slyke method. If, as is practically certain, the ammonia nitrogen was present as NH_4Cl this would be equal to 5.14 gm. By the conventional method of determining sugar by Fehling's solution an equivalent of 18.5 gm. of dextrose was found. In view of our complete ignorance of the nature of the organic constituents of this solution we have no evidence whatever that the reduction of the cupric oxide was caused by any kind of carbohydrate whatever. Making the purely arbitrary assumption, however, that dextrose was present in the above amount we have calculated the nitrogen content of the remaining organic matter as follows. Deducting the sum of the ash, NH_4Cl, and dextrose from the total solids found there would remain 36.76 gm. of organic solids. Further, deducting the nitrogen as ammonia from the total nitrogen, there remains 3.79 gm. of which the amino nitrogen is equal to 53 per cent. The total nitrogen, other than ammonia, is 10.3 per cent of the above corrected solids.

The solution, containing the 82.1 gm. of solids which had been removed by washing the butyl alcohol extracts with water, was examined with the following results.

	gm.	
Total solids	82.1	
Ash	4.43	= 5.4 per cent of the solids.
Nitrogen	6.88	
N as ammonia	0.571	= 8.3 per cent of the nitrogen.
Amino nitrogen	3.323	= 48.3 per cent " " "
Total chlorine	13.26	= 13.63 gm. of HCl.

This solution contained 13.26 gm. of chlorine as determined by adding an excess of Na_2CO_3, evaporating to dryness, and finding chlorine in the ash. As the ash of the untreated substance contained only a slight trace of chlorine we can assume that this fraction of the solids of the alfalfa juice contained 13.63 gm. of HCl.

When 50 cc. of this solution were concentrated *in vacuo* until a solid transparent residue remained, the distillate contained only 0.1140 gm. of HCl equal to 0.684 gm. from the entire 300 cc.

Hence 95 per cent of the HCl was retained by the solids of this solution thereby showing the presence of a large proportion of strongly basic organic substances.

Assuming that the 0.571 gm. of N found as ammonia was present in ammonium salts this would equal 0.734 gm. of NH_4. If HCl + NH_3 + ash = 18.8 gm. is deducted from the 82.1 gm. of solids we have 63.3 gm. of organic solids containing 6.31 gm. of nitrogen, after deducting that found as ammonia.

The organic solids, therefore, contain 10 per cent of nitrogen, of which 52.7 per cent is free amino nitrogen.

It thus appears that by washing the butyl alcohol extract with water one-third of the total nitrogen of the hydrolyzed solids of the alfalfa can be extracted from the butyl alcohol, chiefly, if not wholly, as hydrochlorides of basic substances. Although this solution contains substances which have a strong affinity for HCl only 23.7 per cent of its total nitrogen was precipitated by phosphotungstic acid under the conditions employed in this laboratory for determining basic nitrogen in the products of protein hydrolysis.

The nature of the nitrogenous substances thus extracted is at present being investigated.

PHOSPHORIC ESTERS OF SOME SUBSTITUTED GLUCOSES AND THEIR RATE OF HYDROLYSIS.

By P. A. LEVENE AND G. M. MEYER.

WITH THE ASSISTANCE OF I. WEBER.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

A previous communication contained a report[1] on the constants of hydrolysis of several phosphoric acid esters of glucose. The allocation of the phosphoric acid radical was based on the work of Irvine and Scott[2] on the structure of diacetone glucose. At that time we had overlooked a later publication from Irvine's laboratory in which the previous theory of the structure of diacetone glucose was revised. The positions therein assigned by Macdonald[3] to the acetones are 1, 2- and 5, 6-, hence the benzoyl derivative obtained from it is 1,2- 5,6- diacetone-3-benzoyl glucose.

On this basis, the two phosphoric acid esters, one derived from the diacetone glucose, the other from the diacetone monobenzoyl derivative have the following structure: the former 1,2- 5,6-diacetone-3-phosphoric acid glucose and the latter 1, 2-acetone-3-benzoyl-5- or 6-phosphoric acid glucose.

It seems a strange coincidence that Karrer and Hurwitz[4] also overlooked the work of Macdonald and recently published a paper in which they dealt with the structure of diacetone glucose and assigned to it the same structure which had been formulated by Macdonald.

According to the present formulation the more resistant compound is the one in which the phosphoric acid radical is in position

[1] Levene, P. A., and Meyer, G. M., *J. Biol. Chem.*, 1921, xlviii, 233.

[2] Irvine, J. C., and Scott, J. P., *J. Chem. Soc.*, 1913, ciii, 568.

[3] Macdonald, J. L. A., *J. Chem. Soc.*, 1913, ciii, 1896.

[4] Karrer, P., and Hurwitz, O., *Helvetica Chim. Acta*, 1921, iv, 728.

5 or 6, probably in position 6. This is in harmony with the previous observations on the rate of hydrolysis of 5-phosphoric acid ribose. The latter was found very resistant to hydrolytic agents.

At the time when the allocation 1,2- 3,5 was assigned to the acetones in the diacetone glucose the position 5,6 was assigned to the benzaldehyde in benzylidene glucoside. Hence the respective positions of the inorganic acid in the phosphoric esters of the two sugar derivative should be different. These considerations led us to prepare the substance.

On the basis of the latest formulation the second acetone in the diacetone glucose and the benzaldehyde in the benzylidene monoacetone glucose should each enter into positions 5, 6. Hence the phosphoric acid derivative of diacetone glucose and that of 1,2-acetone and 5,6-benzylidene glucose should both have the phosphoric acid in position 3. To our surprise the constant of hydrolysis of the phosphoric ester obtained from the benzylidene derivative was found $17(10^{-3})$, which is of the same order of magnitude as that of the phosphoric acid derivative obtained from 1,2-acetone-3-benzoyl glucose, whereas a constant of about $54(10^{-3})$ was expected.

A simple explanation for this unexpected result may be found in the assumption that in the process of preparation the benzaldehyde is cleaved off before the phosphoric acid combines with the glucose. This possibility requires special consideration in view of the fact that the product obtained from benzylidene acetone glucose was acetone phosphoric acid glucose. Thus the benzaldehyde group was lost in the process of preparation. There is reason to believe that the cleavage of the benzaldehyde took place after the union with phosphoric acid was accomplished. Had the cleavage of the benzaldehyde been the first step in the operation, then the resulting substance would be 1,2-monoacetone glucose; however, when this compound is acted upon by phosphorous oxychloride a product is obtained which has a constant of hydrolysis $= 44(10^{-3})$. Furthermore, when diacetone glucose is treated with phosphorous oxychloride two esters are obtained, one of diacetone glucose and the second of monoacetone glucose. The constants of hydrolysis of both are practically identical, namely $56(10^{-3})$ and $58(10^{-3})$, thus showing that the position of the phosphoric acid in both is identical and hence

indicating that the cleavage of the acetone took place after the phosphoric acid had united with the glucose.

A *priori* two other alternative explanations are possible. One is that the position of the benzaldehyde in the benzylidene acetone glucose is not on carbon atoms 5 and 6, the second that in the course of the reaction the phosphoric acid radical wanders from position 3 to either 5 or 6. Further investigations will deal with this problem.

The findings on the phosphoric esters of the methylated sugars are also now seen in a different light. The abnormal course of the hydrolysis of 2-phosphoric acid-3,5,6-trimethyl-methyl glucoside is apparently due to the fact that the substance contained a very small admixture of 6-phosphoric acid -2, 3, 5-trimethyl-methyl glucoside. The hydrolysis of the principal substance was undoubtedly completed in the first 60 minutes. Its constant of hydrolysis is not less than $84(10^{-3})$ or $87(10^{-3})$.

The following table contains the rates of hydrolysis of the phosphoric esters of substituted glucoses with special reference to the allocation of the phosphoric acid.

		K
1	5- or 6 (?)-phosphoric acid methyl glucoside (from α-methyl glucoside)...............................	$22(10^{-3})$
2	Mixture of 3-phosphoric acid with 5- or 6-phosphoric acid monoacetone glucose (from monoacetone glucose)......................................	$44(10^{-3})$
3	3-phosphoric acid-1,2- 5,6-diacetone glucose (from diacetone glucose)................................	$56(10^{-3})$
4	3-phosphoric acid-1, 2-monoacetone glucose (by-product from diacetone glucose)....................	$58(10^{-3})$
5	5- or 6-phosphoric acid-3-monobenzoyl-1,2-acetone glucose (from 1,2-monoacetone-3-monobenzoyl glucose)..	$18(10^{-3})$
6	5- or 6-phosphoric acid-1,2-acetone glucose (from 5 by removing benzoyl group)......................	$24(10^{-3})$
7	5- or 6 (?)-phosphoric acid-1,2-acetone glucose (from benzylidene monoacetone glucose)...............	$17(10^{-3})$
8	6-phosphoric acid-2, 3, 5-trimethyl-methyl glucoside.	$44(10^{-3})$
9	2-phosphoric acid-3, 5, 6-trimethyl-methyl glucoside.	$87(10^{-3})$

Benzylidene Monoacetone Glucose.

50 gm. of monoacetone glucose are heated with 300 cc. of freshly distilled benzaldehyde and 50 gm. of anhydrous sodium sulfate at 145°C. for 5 hours. While still warm, the solution is filtered into a distilling flask and the larger part of the benzaldehyde removed by distillation under diminished pressure. When the contents of the flask show a tendency to gelatinize, they are poured into 1 liter of ligroin (80–90°). On vigorous stirring the oily mass soon crystallizes. The crude product is filtered and washed with cold dry ether, in which it is practically insoluble. A nearly pure white product is obtained. Recrystallized several times from absolute alcohol this melts at 141-142°C. The yield is 20 gm.

0.1008 gm. substance: 0.2306 CO_2 and 0.0628 gm. H_2O.

$C_{16}H_{20}O_6$.   Calculated.   C   62.3,   H   6.48.
Found.   "   62.38,   "   6.97.

The substance had the following rotation:

$$[\alpha]_D^{25} = \frac{+ 0.44° \times 100}{2 \times 1} = + 22°.$$

1, 2-Monoacetone-6-Phosphoric Acid Glucose from Benzylidene Monoacetone Glucose.

20 gm. of dried benzylidene monoacetone glucose are dissolved in 75 cc. of dry pyridine and cooled to −20°C. To this is added at once, a solution of 10 gm. of phosphorous oxychloride in 25 cc. of pyridine. The temperature of the reaction mixture does not go above +20°C. and crystals of pyridine hydrochloride settle out immediately on cooling. If the oxychloride solution is added too slowly the reaction is incomplete and only a small quantity of pyridine hydrochloride separates. After allowing the reaction mixture to stand for 1 hour, cold water is added. After the addition of 120 gm. of barium hydroxide, the pyridine is removed by distillation under diminished pressure. The residue is then neutralized with sulfuric acid until just acid to Congo red, shaken for ½ hour with 60 gm. of silver sulfate, and filtered. The filtrate is treated with hydrogen sulfide, the resulting precipitate removed by filtration. After removing the hydrogen sulfide by

a current of air the solution is again made alkaline with barium hydroxide. The excess barium is removed by means of carbon dioxide and the filtrate concentrated under diminished pressure to a syrup. This is taken up in absolute alcohol, filtered, and the barium salt precipitated in a large volume of dry ether. The substance analyzed for a monoacetone phosphoric acid glucose.

0.1104 gm. substance: 0.1156 gm. CO_2, 0.042 gm. H_2O, and 0.0481 gm. ash.
0.2916 "   "   : 0.0746 " $Mg_2P_2O_7$.
0.0972 "   "   : 0.0400 " $BaSO_4$.
$C_9H_{16}O_6H_2PO_3$ Calculated.   C 35.75, H 6.00, P 11.28.
          Found (calculated Ba-free).   " 37.7, " 5.46, " 9.45.

Monoacetone Phosphoric Acid Glucose from Monoacetone Benzylidene Glucose.

7.271 gm. of the barium salt of this substance were dissolved in water and the volume was made up to 50 cc. Of this solution 3 cc., equivalent to 0.03 gm. of P, were put into glass tubes together with 2.1 cc. of N H_2SO_4 and 0.9 cc. of water and sealed. The tubes were heated at 100°C. for the intervals indicated in the following table. The contents of each tube were made up to 100 cc. and the P in 40 cc. portions was determined.

5-6 (?)-Phosphoric Acid-1, 2-Acetone Glucose.

Time.	$Mg_2P_2O_7$ in 40 cc.	Average.	P in $Mg_2P_2O_7$ in 100 cc.	P in free acid.	P of total P.	x	$a - x$	K
min.				per cent	per cent	gm. $Mg_2P_2O_7$	gm. $Mg_2P_2O_7$	$\frac{1}{2}\log\frac{a}{a-x}$
120	0.0018	0.0020	0.0014	0.43	4.6	0 0050	0.1027	0.000176
	0.0022							
240	0.0044	0.0030	0.0030	0.99	10 0	0.0107	0.0970	0.000182
	0.0042							
360	0.0060	0.0055	0.0038	1 19	12 8	0 0138	0.0939	0 000166
	0.0050							
480	0.0072		0.0050	1 57	16 8	0.0181	0 0896	0.000167

$a = 0.1077$

SULFURIC ESTERS OF SOME SUBSTITUTED GLUCOSES AND THEIR RATE OF HYDROLYSIS.

By P. A. LEVENE and G. M. MEYER.

With the Assistance of I. Weber.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

It has been demonstrated in this laboratory that all known glucoproteins are protein derivatives of complex substances which are essentially sulfuric acid esters of disaccharides. It has been observed that individual esters of this group differ in their stability. In some of them the sulfuric acid is removed from the disaccharide very readily, whereas in other compounds it is removed with greater difficulty. *A priori* it seemed possible to explain the differences in stability of individual compounds by the difference in the position of the sulfuric acid on the glucose molecule. In this respect the behavior of the conjugated sulfuric acids resembles that of the conjugated phosphoric acids. In regard to the phosphoric esters of glucose,[1] it was demonstrated experimentally that their resistance is determined by the position of the phosphoric acid on the sugar molecule.

These considerations led us to synthesize two sulfuric acid sugar derivatives, one from diacetone glucose and the other from 1,2-acetone-3-benzoyl glucose. The first should yield a substance with the sulfuric acid attached to carbon atom 3, the second to either carbon atom 5 or 6.

For the preparation of sulfuric acid esters of glucose several methods have been recommended by Neuberg and Pollak,[2] and Neuberg and Liebermann.[3] They employed for the purpose either pyrosulfates or chlorosulfonic acid. Neuberg and Pollak have also

[1] Levene, P. A., and Meyer, G. M., *J. Biol. Chem.*, 1922, liii, 431.

[2] Neuberg, C., and Pollak, H., *Biochem. Z.*, 1910, xxvi, 515.

[3] Neuberg, C., and Liebermann, L., *Biochem. Z.*, 1921, cxxi, 326.

mentioned that sulfuryl chloride may be used for this purpose.
However, for the use of this reagent no experimental directions
are given by them. Helferich[4] has also worked with sulfuryl
chloride and obtained a dichlorohydrinsulfate. In the present
experiments sulfuryl chloride was employed. The success of the
synthesis depends largely on the temperature maintained during
the operation. It was found advantageous to add the solution
of sulfuryl chloride in chloroform to a pyridine solution of the
sugar derivative cooled to $-10°C.$, and to allow the temperature to
rise to about 30°C. The details of the preparation are given in
the experimental part.

Regarding the rates of hydrolysis it was found that the ester
having the sulfuric acid in position 5 or 6 was more stable than
the one having the acid in position 3 as may be seen from the
following table.

$$K = \tfrac{1}{2}\log\frac{a}{a-x}$$

3-sulfuric acid-1, 2- 5, 6-diacetone glucose .... 60 (10^{-3})
5- or 6-sulfuric acid-1, 2-acetone glucose .... 40 (10^{-3})

This is in harmony with the views expressed in the publi-
cations from this laboratory on the structure of chondroitin and
mucoitin sulfuric acids.

EXPERIMENTAL.

Sulfuric Acid Diacetone Glucose.

A solution of 10 gm. of diacetone glucose in 30 cc. of dry
pyridine, is cooled to $-10°C.$ To this is added a solution of
2.7 cc. of sulfuryl chloride in 25 cc. of dry chloroform, also
cooled to $-10°C.$ The temperature rises to 30°C., and the
reaction product is allowed to stand at room temperature over
night. The product is then dark red and contains no pre-
cipitate. Water and 60 gm. of barium hydroxide are then added
and the chloroform and pyridine removed by distillation under
diminished pressure. The solution is treated with sulfuric acid
until it turns acid to Congo red and is shaken for $\tfrac{1}{2}$ hour with 30
gm. of silver sulfate. The excess silver is removed by hydrogen
sulfide, which is then removed by a current of air. The excess of

[4] Helferich, B., *Ber. chem. Ges.*, 1921, liv, 1082.

barium is removed by means of carbon dioxide and the filtrate is concentrated to dryness under diminished pressure. The residue is dissolved in a small amount of absolute alcohol, the solution is filtered and precipitated in a large volume of dry ether.

For purification the precipitate is redissolved in absolute alcohol and reprecipitated in ether, this process is repeated several times.

10 gm. of diacetone glucose yield 5 gm. of the barium salt of sulfuric acid diacetone glucose.

The substance does not reduce Fehling's solution until after hydrolysis. Barium chloride, also, produces no precipitate in an aqueous solution of the substance. However, a precipitate of barium sulfate is formed after the substance is hydrolyzed with hydrochloric acid.

The elementary composition of the substance is not so good as is desired. However, taking into consideration the amorphous nature of the substances and their great solubility, the result may be regarded as satisfactory.

0.1096 gm. substance: 0.1334 gm. CO_2 and 0.0454 gm. H_2O.
0.2766 "   "   : 0.1320 " $BaSO_4$ (S determination).
0.0922 "   "   : 0.0254 "   "   (Ba   "   ).
$(C_{12}H_{20}O_9S)_2$ Ba. Calculated. C 35.5, H 4.66, S 7.88, Ba 16.85.
                    Found.   " 33.19, " 4.63, " 6.55, " 16.21.

5- or 6 (?)-Sulfuric Acid-Monoacetone Glucose from 3-Benzoyl-1, 2-Monoacetone Glucose.

Benzoyl monoacetone glucose (10 gm.) dissolved in 30 cc. of dry pyridine was reacted on with 2.7 cc. of sulfuryl chloride dissolved in 25 cc. of chloroform, as previously described under sulfuric acid diacetone.

The final residue was dissolved in a small quantity of hot absolute alcohol. On cooling barium benzoate crystallized. This was filtered off and the filtrate precipitated in a large volume of dry ether. This process was repeated several times.

The substance obtained analyzed for sulfuric acid monoacetone. It does not reduce Fehling's solution. An aqueous solution of the substance gives no precipitate with barium chloride until after hydrolysis with acid.

0.1062 gm. substance: 0.1136 gm. CO_2, 0.0352 gm. H_2O, and 40.3 gm. ash.
0.2912 "   "   : 0.1534 " $BaSO_4$ (S   determination).
0.0971 "   "   : 0.0390 "   "   (Ba   "   ).
$(C_9H_{15}O_9S)_2$ Ba.   Calculated.   C 29.4, H 4.3, S 8.7, Ba 18.35.
                    Found.   " 29.17 " 3.7, " 7.23 "   23.63.

Sulfuric Acid Diacetone Glucose.

3.817 gm. of the barium salt of this substance were dissolved in a little water and the volume was made up to 25 cc.   Of this solution 3 cc., equivalent to 0.031 gm. of S, were put into glass tubes together with 1.68 cc. of N HCl and 4.32 cc. of H_2O.   After the tubes were sealed they were heated at 75°C. for the time intervals indicated in the following table.   The sulfuric acid was determined as $BaSO_4$.

Time.	$BaSO_4$	S in $BaSO_4$.	S in free acid.	S of total S.	x	$a - x$	K
min.	gm.	gm.	per cent	per cent	gm. $BaSO_4$	gm. $BaSO_4$	$\frac{1}{2} \log \frac{a}{a-x}$
60	0.0153	0.0021	0.547	7.00	0.0153	0.2032	0.00053
240	0.0620	0.0085	2.22	28.4	0.0620	0.1565	0.00060
360	0.0857	0.0118	3.07	39.2	0.0857	0.1328	0.00060

$a = 0.2184$

Sulfuric Acid Monoacetone Glucose.

3.481 gm. of the barium salt of this substance were dissolved in a little water and the volume was made up to 25 cc.   Of this solution 3 cc., equivalent to 0.031 gm. of S, were put into glass tubes together with 1.94 cc. of N HCl and 4.06 cc. of H_2O.   After the tubes were sealed they were heated at 75°C. for the time intervals indicated in the following table.   The sulfuric acid was determined as $BaSO_4$.

Time.	$BaSO_4$	S in $BaSO_4$.	S in free acid.	S of total S.	x	$a - x$	K
min.	gm.	gm.	per cent	per cent	gm. $BaSO_4$	gm. $BaSO_4$	$\frac{1}{2} \log \frac{a}{a-x}$
120	0.0119	0.0016	0.502	5.45	0.0119	0.2066	0.00040
240	0.0480	0.0066	2.02	22.0	0.0180	0.1705	0.00044
360	0.0622	0.0085	2.62	28.5	0.0622	0.1563	0.00040

PREPARATION AND ANALYSIS OF ANIMAL NUCLEIC ACID.

By P. A. LEVENE.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

The preparation of a pure animal nucleic acid remained a diffi-
cult task notwithstanding the fact that several methods have been
recommended in recent years. A year ago the present writer
made an effort to prepare animal nucleic acid by his picric acid
method, slightly modifying the details of the procedure. The
result was quite satisfactory when small quantities of the material
were prepared by the writer personally. However, when the
preparation of the material was left in the hands of technical
assistants the product obtained by them was rather impure and
besides had a brownish color which made the measurement of its
optical activity very difficult. A new effort was made to work
out an easy and reliable process. Preliminary to this attempt we
tested all the recently published methods of W. Jones,[1] R. Feul-
gen,[2] and E. J. Baumann.[3] None proved satisfactory, since every
sample prepared by any one of these methods gave a very marked
biuret reaction. That the authors themselves obtained satis-
factory results by their respective methods is not doubted, but
we affirm that such results, to say the least, are not constant.
Material prepared by the method of Jones[1] by a French firm also
proved unsatisfactory.

After several trials we finally modified our older method by
substituting colloidal iron for the picric acid.

The details of the process as carried out at present are as
follows: The glands (10 lbs.) are dissected free from fat, ground
in a chopping machine, and transferred into 5 liters of water,

---

[1] Jones, W., Nucleic acids; their chemical properties and physiological
conduct, New York, 2nd edition, 1920.

[2] Feulgen, R., *Z. physiol. Chem.*, 1917–18, ci, 296.

[3] Baumann, E. J., *Proc. Soc. Exp. Biol. and Med.*, 1919–20, xvii, 118.

containing 250 gm. of sodium hydroxide. The mixture is boiled
for 35 minutes and then neutralized with acetic acid. 50 cc. of a
colloidal iron solution (iron dialyzed, 5 per cent Fe_2O_3, Merck)
are added and the solution is filtered and allowed to stand over
night. To the filtrate is then added a double volume of methyl
alcohol containing 2 per cent of hydrochloric acid. The pre-
cipitate thus formed is filtered off and washed with methyl alcohol
until the filtrate is free from hydrochloric acid.

This process has been applied to the preparation of nucleic
acid from thymus gland, spleen, kidney, pancreas, and liver.
The average respective yields from 10 lbs. of gland were: thymus,
150.0 gm.; spleen, 40.0 gm.; kidney, 25.0 gm.; pancreas, 35.0 gm.;
and liver, 18.0 gm.

From the first four organs the first precipitation yields a ma-
terial which is either entirely free, or contains barely detectable
traces, of biuret-giving substances. In the last case one repre-
cipitation with alcohol containing 2 per cent hydrochloric acid
suffices to purify the material. The material obtained from the
liver contains a marked proportion of glycogen. Such prepara-
tions are readily purified by dissolving them in water with the
aid of a minimum amount of alkali, slightly acidulating with
hydrochloric acid, and precipitating the nucleic acid with a
20 per cent solution of cupric chloride. The precipitate is washed
with water, suspended in alcohol containing 2 per cent hydro-
chloric acid, and carefully triturated until the larger part of the
copper is recovered. The precipitate is then dissolved in water,
adding a minimum amount of alkali, and the nucleic acid is
precipitated by a double volume of alcohol containing 2 per cent
hydrochloric acid. When this process fails, the crude nucleic acid
may be dissolved in water with the aid of a minimum amount of
alkali and precipitated with hydrochloric acid. This precipitate is
then redissolved in water, by the addition of a minimum amount
of alkali and to the solution an equal volume of alcohol, containing
2 per cent of hydrochloric acid, is added.

The following table contains the results of the analysis of
various samples obtained by this process:

	C	H	N	P
Thymus gland......................	36 72	4 58	14.59	9 05
Spleen............................	36 21	4 30	14.54	8 94
Kidney...........................	36 51	4 17	14 07	8.74
Pancreas.........................	36 34	4.40	14 37	9.10
Liver............................	35 69	4 05	15.25	9 05
Calculated for a hexose tetra-nucleotide........................	36 30	4 19	14.79	8.73

The agreement of the analytical results with the theory is quite satisfactory. However, not too much importance should be attached to it, since the elementary composition of amorphous substance has only a relative theoretical importance. Besides, not all samples gave identical analytical results. Often the original material contained 10 per cent of phosphorus, and about 12 per cent of nitrogen, but showed little deviation from the theory in the content of carbon and hydrogen. On the other hand, the table is very important in showing that nucleic acids derived from different organs do not vary in their elementary composition notwithstanding the claims to the contrary recently expressed by Feulgen and others.

Also as regards the content of purine bases, the acids from the various organs seem to show only such variations as could be expected from the degree of accuracy of the analytical methods. Thus the theory of the tetranucleotide requires for adenine picrate 27.10 per cent and for guanine 10.6 per cent purine bases. The results of the analysis were as follows:

	Adenine picrate.	Guanine.
Thymus gland...........................	26 6	11.8
Spleen.................................	26.6	13.0
Kidney.................................	23 3	12.6
Pancreas...............................	23 0	11 5
Liver..................................	30.0	11.8

ANALYTICAL PART.

The hydrolysis of the nucleic acids for the purpose of estimating the purine bases was carried out in the same way as described in a previous article, with the exception that instead of absolute

methyl alcohol, one containing 5 per cent of water was employed.
The nucleic acid (50 gm.) is suspended in 500 cc. of 95 per cent
methyl alcohol and hydrogen chloride gas is passed for 2 hours.
The acid soon dissolves and gradually the hydrochlorides of the
bases settle out.   To complete the separation the reaction product
is allowed to stand over night.

Separation and Purification of the Purine Bases.—After a
scrutiny of the analytical data on the nucleic acids published by
different writers one is left with the impression that the workers
encountered difficulties in obtaining the bases in analytically
pure condition.   This is particularly true regarding guanine.   In
part this difficulty arose from the method of hydrolysis, in part
also from the method of isolating the bases.   It is possible, how-
ever, to prepare the bases in pure condition in a very short time.
The process employed in the course of this work was the following:

1. *Separation of Adenine from Guanine.*—The hydrochlorides
are dissolved in hot water and the solution is neutralized with
sodium hydroxide until neutral to Congo red.   The guanine then
precipitated out.   A precipitate is then formed which consists
practically of pure guanine, slightly contaminated with adenine.
In order to remove this it is again dissolved in dilute hydrochloric
acid and again precipitated with sodium hydroxide.   The two
filtrates are combined and the adenine is precipitated in the form
of the picrate.   In the course of the present work the crude
adenine picrate and the crude guanine were dried to constant
weight.   The crude adenine picrate contains about 28 per cent
of nitrogen whereas 29.3 per cent is required by theory and the
guanine contained about 40 per cent of nitrogen whereas 46.35 per
cent is required by theory.

2. *Purification of the Base.*—Adenine picrate is obtained
analytically pure by one recrystallization from 25 per cent solution
of acetic acid.   The crude picrate (10 to 12 gm.) is suspended in
1 liter of the acid, and boiled until dissolved.

Guanine is obtained analytically pure in the following manner.
The crude material is dissolved in boiling dilute sulfuric acid and
precipitated by means of silver sulfate.   The precipitate of guanine
silver sulfate is filtered off while the reaction product is still hot.
The silver salt is decomposed by means of hydrochloric acid and
the clear filtrate from silver chloride is neutralized with 10 per

cent of sodium hydroxide. Free guanine is thus precipitated. On some occasions it was found necessary to repeat the silver precipitation process.

Analysis of Individual Nucleic Acids.

Thymus Gland.

0.1184 gm. substance: 0.1384 gm. CO_2 and 0.0420 gm. H_2O.
0.1736 "   "   required (Kjeldahl) 18.10 cc. 0.1 N acid.
0.2605 "   "   : 0.0846 gm. $Mg_2P_2O_7$.
            Found.   C 36.72, H 4.58, N 14.59, P 9.05.

Bases.—45.0 gm. of the dry material yield 12.0 gm. (26.6 per cent) of crude adenine picrate and 5.3 gm. (11.8 per cent) of crude guanine, containing 40 per cent nitrogen.

Adenine picrate analyzed as follows:

0.1000 gm. required (Kjeldahl, reduction with zinc) 20.75 cc. 0.1 N acid.
        $C_5H_5N_5C_6H_2(NO_2)_3$ OH + H_2O.   Calculated. N 29.31.
                                         Found.   " 29.05.

Guanine was analyzed as the free base.

0.0983 gm. substance required (Kjeldahl) 32.25 cc. 0.1 N acid.
        $C_5H_5N_5O$. Calculated. N 46.35.
                    Found.   " 45.93.

Spleen Nucleic Acid.

0.1006 gm. substance: 0.1444 gm. CO_2 and 0.0418 gm. H_2O.
0.1795 "   "   required (Kjeldahl) 18.65 cc. 0.1 N acid.
0.2693 "   "   : 0.0864 gm. $Mg_2P_2O_7$.
            Found.   C 36.21, H 4.30, N 14.54, P 8.94.

Bases.—45.0 gm. of the dry acid gave 12.0 gm. (26 per cent) of crude adenine picrate and 6.0 gm. (13.3 per cent) of crude guanine, having 39.2 per cent of nitrogen.

Adenine was purified by recrystallization and analyzed as follows:

0.1000 gm. substance: (Dumas) 26 cc. nitrogen at 26°C., 756 mm.
        $C_5H_5N_5$. $C_6H_2(NO_2)_3$ OH+H_2O. Calculated. N 29.31.
                                         Found.   " 29.51.

Guanine was analyzed both as the free base and as the picrate.

0.0988 gm. substance required (Kjeldahl) 32.35 cc. 0.1 N acid.
$C_5H_5N_5O$.   Calculated.   N 46.35.
Found.   " 45.84.

This substance was dissolved in water on addition of the required amount of 10 per cent sodium hydroxide. An excess of picric acid was then added. The picrate settled out gradually in the form of long needles and analyzed as follows:

0.1000 gm. substance: (Dumas) 24.2 cc. nitrogen at 23°C., 763 mm.
$C_5H_5N_5O$. $C_6H_2(NO_2)_3$ OH+H_2O.   Calculated.   N 28.13.
Found.   " 28.02.

Kidney Nucleic Acid.

0.0992 gm. substance: 0.1312 gm. CO_2 and 0.0378 gm. H_2O.
0.1693 "   "   required (Kjeldahl) 16.95 cc. 0.1 N acid.
0.2539 "   "   : 0.0834 gm. $Mg_2P_2O_7$.
Found. C 36.06, H 4.26, N 14.01, P 9.15.

Bases.—42.0 gm. of the substance yielded 9.8 gm. (23.3 per cent) of adenine picrate and 5.3 gm. (12.6 per cent) of crude guanine (N = 39.05 per cent).

Adenine picrate was purified by recrystallization and analyzed as follows:

0.1000 gm. substance: (Dumas) 25.3 cc. nitrogen gas at 23°C., 758 mm.
$C_5H_5N_5$. $C_6H_2(NO_2)_3$ OH+H_2O.   Calculated.   N 29.31.
Found.   " 29.10.

Guanine was analyzed as the sulfate.

0.0967 gm. of the dry substance required (Kjeldahl) 22.65 cc. 0.1 N acid.
$(C_5H_5N_5O)_2$ H_2SO_4.   Calculated.   N 34.99.
Found.   " 34.96.

Pancreas Nucleic Acid.

0.1037 gm. substance: 0.1382 gm. CO_2 and 0.0408 gm. H_2O.
0.1866 "   "   required (Kjeldahl) 19.15 cc. 0.1 N acid.
0.2799 "   "   : 0.0914 gm. $Mg_2P_2O_7$.
Found. C 36.34, H 4.40, N 14.37, P 9.10.

Bases.—The crude substance (35.0 gm.) gave 8.0 gm. (23 per cent) of crude adenine picrate and 4.0 gm. (11.5 per cent) of crude guanine.

Adenine picrate was recrystallized once and analyzed as follows:

0.1000 gm. substance: (Dumas) 26 cc. nitrogen at 26°C., 752 mm.
$C_5H_5N_5$. $C_6H_2(NO_2)_3$ OH+H_2O. Calculated. N 29.31.
Found. " 29.33.

Guanine was identified as the free base.

0.0979 gm. substance required (Kjeldahl) 32.10 cc. 0.1 N acid.
$C_5H_5N_5O$. Calculated. N 46.35.
Found. " 45.90.

Liver Nucleic Acid.

0.1094 gm. substance: 0.1432 gm. CO_2 and 0.0396 gm. H_2O.
0.1800 " " required (Kjeldahl) 18.55 cc. 0.1 N acid.
0.2000 " " : 0.0924 gm. $Mg_2P_2O_7$.
Found. C 35.69, H 4.05, N 15.25, P 9.05.

Bases.—The crude acid (33 gm.) gave 9.0 gm. (30 per cent) of adenine picrate and 3.9 gm. (11.8 per cent) of crude guanine (by error was not analyzed).

Adenine picrate was recrystallized once and analyzed as follows:

0.1000 gm. substance: (Dumas) 26.4 cc. nitrogen at 29°C., 753 mm.
$C_5H_5N_5$. $C_6H_2(NO_2)_3$ OH+H_2O. Calculated. N 29.31.
Found. " 29.57.

Guanine was analyzed as the free base.

0.0963 gm. substance required (Kjeldahl) 31.75 cc. 0.1 N acid.
$C_5H_5N_5O$. Calculated. N 46.35.
Found. " 46.15.

BENZYLIDENE-ETHYL-CHITOSAMINATE AND BENZYL-IDENE-ETHYL-DIAZOGLUCONATE (MANNONATE).

By P. A. LEVENE.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

Levene and La Forge[1] observed that benzylidene-ethyl-chito-saminate hydrochloride on cautious treatment with sodium nitrite, is converted into the corresponding diazo derivative. On the other hand, it has been known that chitosaminic acid and chitosamine are converted by the same reagent, not into a mixture of two epimeric deaminized derivatives, but each into a single derivative. To Levene and La Forge it seemed possible to regard the diazo derivative as an intermediate substance in the process of deamination. However, there existed no experimental proof showing the transformation of the diazo esters of the sugar acids into a single sugar acid and not into a pair of epimers.

There also existed no experimental data regarding the character of the substances which would result from the substitution of the diazo group by hydrogen chloride or bromide.

In the present work the diazo compound was hydrolyzed and the resulting hydroxy-acid identified, on the other hand, the diazo compound was converted into the bromo and chloro compounds and the chloro derivative again converted into a 2-amino-hexonic acid.

On hydrolysis of the diazo derivative with dilute acetic acid the formation of only one derivative was observed; namely, benzylidene-ethyl-gluconate. This was identified in the form of saccharic acid. However, in one experiment, anhydrosaccharic acid was isolated. The bromo and chloro derivatives were prepared each with a constant melting point and constant specific

[1] Levene, P. A., and La Forge, F. B., *J. Biol. Chem.*, 1915, xxi, 345.

rotation, showing that in each instance only one substance and not a pair of epimers had been formed. The latter conclusion is further substantiated by the fact that the chloro derivative on treatment with ammonia gave but one chitosaminic acid (2-amino-mannonic) and not a mixture of two-aminohexonic acids. One may be inclined to explain these reactions by the presence in the molecule of three asymmetric carbon atoms, on the other hand, this asumption is not bindng since Fischer obtained two epimeric halogen derivatives, when bromine or chlorine was added to glucal.

Regarding the direction of the rotation of carbon atom 2 in the derivatives of the diazo compound, the following was observed. On hydrolysis of the compound with dilute acids a substance resulted in which the rotation of the carbon atom 2 was in the opposite direction from that of the carbon atom 2 of chitosaminic acid. On the other hand, in the bromo, chloro, and amino derivatives, the rotation of the carbon atom 2 was the same as in the original chitosaminic acid. It is here accepted that in the chloro and bromo derivative the direction of the rotation of the carbon atom 2 determines the direction of rotation of the acid. Hence for the present for the chloro and bromo derivatives, the configuration of mannonic acid is assumed. On this assumption chitosaminic acid passes through the diazo derivative into the chloro derivative and back into the amino-acid apparently without Walden inversion. On the other hand, deamination through only the diazo derivative undergoes the Walden inversion in the same way as on direct deamination.

In connection with the Walden inversion it is interesting to note that it occurs in this instance both in the acid and in its ester. In the majority of amino-acids according to the observation of Fischer, the inversion occurs only in the acid and not in the ester. In the amino-acids where acid and ester both gave rise to the same hydroxy-acid, Fischer's original view was that no inversion occurred in either, and later he reversed his opinion accepting an inversion in both. In the present instance chitosaminic acid and ester give a hydroxy-acid in which the carbon atom 2 rotates to the right, whereas chitosamine leads to a hydroxy-acid in which the carbon atom 2 rotates to the left. On the basis of considerations discussed in another article it was as-

sumed that the inversion occurred in chitosaminic acid, hence it also occurs in the ester. This fact is a corroboration of the later theory of Fischer.

The set of reactions here described are represented by the following graphic expression:

I.   II.   III.   IV.   V.

In the course of the work some derivatives were prepared which have no direct bearing on the present problem, but eventually they may become of theoretical value. These compounds are: Benzylidene-chitosaminic acid, benzylidene-ethyl-chitosaminate (this was reconverted into its hydrochloride), benzylidene-acetone-ethyl-chitosaminate, benzylidene-1-ethyl-2, 3-anhydrogluconate (mannonate), and benzylidene-ethyl-desoxygluconate (mannonate). The last may be a mixture of gluconate and mannonate.

Benzylidene-1-amino-2,3-dehydrogluconate   (mannonate)   and benzylidene-1-amino-2-chloromannonate were also prepared.

The first three substances were obtained in the process of preparation of free benzylidene-ethyl-chitosaminate. In one phase of the work it seemed as if the halogen derivative of the diazo compound was readily converted into the free amino derivative, and hence it was desirable to compare the substance obtained from the diazo compound with that from benzylidene-ethyl-chitosaminate hydrochloride.

The benzylidene-acetone-ethyl-chitosaminate was obtained accidentally, when it was attempted to recrystallize benzylidene-ethyl-chitosaminate from acetone. The condensation took place in the short time required to bring the original substance in solution. It is remarkable that the acetone is cleaved off as readily as it is condensed with the benzylidene-ethyl-chitosaminate. Benzylidene-chitosaminic acid was obtained as a by-product in the process of preparation of its ester.

Benzylidene-1-ethyl-2,3-anhydrogluconate (mannonate) is obtained almost instantly when an alcoholic solution of benzylidene-ethyl-chitosaminate is poured into aqueous ammonia and the solution cooled to 0°C. This derivative apparently retains the enolic structure

$$\begin{array}{c} CH_2 \cdot CH \cdot CHOH \cdot CHOH \cdot CH = CHO \cdot COOC_2H_5 \\ | \qquad | \\ O \qquad O \\ \diagdown \diagup \\ CHC_6H_5 \end{array}$$

and not the structure

$$\begin{array}{c} CH_2 \cdot CH \cdot CHOH \cdot CHOH \cdot CH_2 \cdot CO \cdot COOC_2H_5 \\ | \qquad | \\ O \qquad O \\ \diagdown \diagup \\ CHC_6H_5 \end{array}$$

since with phenylhydrazine it did not form a hydrazone, and furthermore in the presence of palladium it was readily hydrogenated giving the following substance:

$$\begin{array}{c} CH_2 \cdot CH \cdot CH(OH) \cdot CH(OH) \cdot CH_2 \cdot CHOH \cdot COOC_2H_5 \\ | \qquad | \\ O \qquad O \\ \diagdown \diagup \\ CHC_6H_5 \end{array}$$

(The position of the desoxy carbon atom may be either 2 or 3.)

It is not known whether this is a single substance or a mixture of two epimers. Thus attempts to convert the bromo derivatives into benzylidene-chitosaminic ester failed. The attempt to convert the free bromomannonic acid into chitosaminic acid was also not successful.

Benzylidene-1-amino-2-chloromannonate is formed when an alcoholic solution of the chloro ester is poured into an excess of aqueous ammonia and the solution is allowed to crystallize. The same substance is formed when the chloro ester is dissolved in alcohol containing ammonia gas. On the contrary, if an alcoholic solution of the chloro ester added to aqueous ammonia is heated at 95°C. for 10 hours in a sealed tube the product is benzylidene-ethyl-chitosaminate.

Since in the course of the reaction some decomposition takes place it is more conveniently identified as chitosaminic acid.

EXPERIMENTAL.

Benzylidene-Ethyl-Chitosaminate Hydrochloride.—The substance was prepared under conditions previously described. For purification it was dissolved in methyl alcohol. To the solution dry ether was added until crystallization was complete. The substance melted at 200°C. (uncorrected) and analyzed as follows:

0.1972 gm. substance: (Kjeldahl) 5.65 cc. 0.1 N acid.
0.1972 " " : (Volhard) 5.6 " 0.1 N silver nitrate.
0.020 " " : (a) (Van Slyke) in 4 min. 0.38 cc. nitrogen at 22°C., 753.4 mm.
0.020 gm. substance: (b) (Van Slyke) in 30 min. 0.59 cc. nitrogen at 22°C., 753.4 mm.

$C_{15}H_2NO_7HCl.$ Calculated. N 4.04, Amino N 4.04, Cl 10.94.
Found. " 4.01, " " (a) 1.06, " 10.07.
" " (b) 1.65.

The substance had the following rotation:

$$[\alpha]_D^{20} = \frac{-0.30° \times 100}{1 \times 1} = -30°$$

Benzylidene-Ethyl-Chitosaminate and Benzylidene-Chitosaminic Acid.—The hydrochloride (40.0 gm.) was dissolved in water (150 cc.) and an excess of an aqueous 1.0 N sodium hydroxide

solution (140 cc.) was added. On scratching the walls of the
container the solution nearly solidifies into a mass consisting of
curved needles. The precipitate was filtered on a suction funnel
and washed a few times with water. The precipitate was dried
under diminished pressure over sulfuric acid until practically
dry. The yield of the dry product was about 20.0 gm. The sub-
stance consisted principally of the free benzylidene-chitosaminic
ester with a small admixture of benzylidene-chitosaminic acid.
The filtrate contained a larger quantity of the latter substance.
This crystallized out on standing in the form of large crystalline
plates. The separation of the benzylidene ester from the acid
was accomplished in the following way: The dried substance
(1 part) was taken up in boiling 98.5 per cent alcohol (4 parts)
and filtered. The insoluble part consisted of the benzylidene-
chitosaminic acid. The filtrate contained the ester. On stand-
ing it crystallized out in long prisms. For analysis it was re-
crystallized twice from small volumes of 98.5 per cent alcohol.
It melted at 120°C. (corrected) and analyzed as follows:

0.0770 gm. substance: 0.1638 gm. CO_2 and 0.0432 gm. H_2O.
0.1288   "   "   required (Kjeldahl) 4.45 cc. 0.1 N acid.
    $C_{15}H_{20}NO_6$. Calculated. C 57.85, H 6.80, N 4.50.
                Found   " 57.94, " 6.28, " 4.83.
The substance in methyl alcoholic solution had the following rotation:

$$[\alpha]_D^{20} = \frac{-0.50° \times 100}{1 \times 1} = -50°$$

The benzylidene-chitosaminic acid was purified by dissolving
in hot water and adding 98.5 per cent alcohol to slight opalescence.
On standing the substance crystallized in large prismatic plates.
After two or three crystallizations the substance analyzed cor-
rectly. It melted at 230°C. (uncorrected) and analyzed as
follows:

0.1084 gm. substance: 0.2008 gm. CO_2 and 0.0594 gm. H_2O.
0.1859   "   "   required (Kjeldahl) 6.55 cc. 0.1 N acid.
0.0186   "   "   : (Van Slyke) 1.75 cc. nitrogen at 24.0°C., 765 mm.
    $C_{13}H_{17}NO_6$. Calculated. C 55.09, H 6.05, N 4.94, NH_2 4.94.
                Found   " 55.40, " 6.19, " 4.93, "   4.92.
The substance had in aqueous solution the following optical rotation:

$$[\alpha]_D^{20} = \frac{-0.28° \times 100}{1 \times 1} = 28°$$

Benzylidene-Acetone-Ethyl-Chitosaminate.—This substance was obtained, incidentally, in an experiment aiming to separate benzylidene-chitosaminic acid from its ester. The crude material (40.0 gm.) was suspended in dry acetone and digested on a boiling water bath. A part (3.0 gm.) remained undissolved. This substance proved to be the benzylidene-chitosaminic acid. The mother liquor was allowed to stand in a vacuum desiccator over sulfuric acid. Soon very large prismatic crystals began to form on the edge of the liquid and after a few days crystallization seemed complete. The yield was about 20 gm. From the mother liquor on standing, further crystallization took place. For purification the material may be recrystallized either from absolute alcohol or from acetone. The substance melts sharply at 128°C. It analyzed as follows:

0.1134 gm. substance. 0.2380 gm. CO_2 and 0.732 gm. H_2O.
0.1986 "   "   required (Kjeldahl) 5.55 cc. 0.1 N acid.
0.020   "   "   : (Van Slyke) after 30 min., 0.87 cc. nitrogen at 24°C., 760 mm.

$C_{18}H_{25}NO_6$.   Calculated.   C 61.50, H 7.18, N 3.98.
              Found.   " 61.37, " 7.22, " 3.91.

The optical rotation of the substance was:

$$[\alpha]_D^{20} = \frac{-0.70° \times 100}{1 \times 1} = -70°$$

The substance is readily reconverted into the hydrochloride of benzylidene-ethyl-chitosaminate. It (2.0 gm.) was dissolved in 20 cc. of dry methyl alcohol, to this solution 20 cc. of 0.6 N hydrochloric acid in dry ether were added, then dry ether until slight opalescence. On standing a substance crystallized which melted at 202°C. (uncorrected) and analyzed as follows:

0.1072 gm. substance: 0.2044 gm. CO_2 and 0.0626 gm. H_2O.
0.1932 "   "   required (Kjeldahl) 5.55 cc. 0.1 N acid.
$C_{15}H_{22}NO_6HCl$.   Calculated.   C 51.72, H 6.34, N 4.04.
                  Found.   " 51.75, " 6.50, " 3.99.

Diazo Derivative of Benzylidene-Ethyl-Chitonate.—The original method for the preparation of the diazo ester was slightly modified. The hydrochloride of benzylidene-ethyl-chitosaminate (20 gm.) is dissolved in 250 cc. of water and chilled to the same temperature. The two solutions are combined and to

the resulting solution glacial acetic acid (30.0 cc.) is added. The
contents of the flask are practically solidified. The product at
this phase seems colorless. When filtered off, however, it was a
light yellow. The product is dissolved in ether and the ethereal
solution is washed (four times) in a separatory funnel with a cold
solution of sodium carbonate and then (three times) with water.
The ethereal solution is dried by means of anhydrous sodium
sulfate and concentrated under diminished pressure to a volume
of about 35 cc., then transferred into an evaporating dish which
is placed in a vacuum desiccator over sulfuric acid. The solution
is evaporated to dryness leaving a light yellow mass which is
readily pulverized. The powder is exhaustively extracted with
low boiling petroleum ether and is then analytically pure.

The nitrogen estimation is easily carried out by the process
described by Levene and Mikeska.[2]

Several samples were analyzed in this manner with the follow-
ing results:

Sample 1. 0.020 gm. substance: 1.60 cc. nitrogen at 26°C., 760 mm.
"   2. 0.020 "   "   : 1.57 "   "   " 26° " 760 "
"   3. 0.020 "   "   : 1.54 "   "   " 26° " 760 "
          $C_{15}H_{18}O_6N_2$. Calculated.   N 8.69.
                    Found, Sample 1.   " 8.88.
                         "   "   2.   " 8.71.
                         "   "   3.   " 8.55.

The optical rotation of the substance was the following:

$$[\alpha]_D^{20} = \frac{-1.00° \times 100}{2 \times 1} = -50°$$

Hydrolysis of the Diazo Derivative.—The product resulting from
hydrolysis of the diazo derivative depends on the conditions of
the reaction. If the reaction takes place in the absence of water
or in organic solvents containing only a small proportion of water
the product is apparently ethyl-2,3-anhydrogluconate, otherwise
ethyl-gluconate or ethyl-1,4-anhydrogluconate is formed as the
principal product. It was found difficult to isolate and identify
the original reaction product, hence this was further oxidized by
means of nitric acid. In the conditions in which only the unsatur-
ated acid formed the product of oxidation was mesotartaric acid,
under other conditions the product of oxidation was either

[2] Levene, P. A., and Mikeska, L. A., *J. Biol. Chem.*, 1922, lii, 485.

saccharic or anhydrosaccharic acid. The conditions of reaction were as follows:

The diazo compound (20 gm.) was suspended in 400 cc. of distilled water and glacial acetic acid (20.0 gm.) was added. The flask is placed near a hot water bath and from time to time warmed on the water bath so as to maintain a continuous but gentle evolution of nitrogen gas. The reaction was considered completed when a dark yellow oil (benzaldehyde) settled out on the bottom of the flask. The oil is separated in a separatory funnel, the aqueous portion is extracted with ether and concentrated to nearly dryness under diminished pressure. The residue is dissolved in water and again concentrated under diminished pressure. The operation is repeated four times. In this manner the greatest part of the acetic acid is removed. The final residue is taken up in 40.0 cc. of water, an equal volume of concentrated nitric acid is added, and the solution is allowed to stand over night. It is then transferred to a clock-glass and evaporated to dryness on a water bath. The residue is dissolved in nitric acid, diluted with an equal volume of water, and the solution evaporated to dryness. The residue is then dissolved in water and the process repeated. The final product is dissolved in water, again shaken out with ether, and finally converted into the calcium salt.

The yield of the calcium salt is about 5 to 6 gm. from 100 gm. of the diazo compound. In the earlier experiments the calcium salt was purified by repeatedly dissolving it in water containing the requisite amount of oxalic acid and reconverting it into the calcium salt until a product with a maximum optical rotation of about $[\alpha]_D^{20} = + 24.0°$ was obtained. Under these conditions anhydrosaccharic acid was obtained.

When the calcium salt was only once recrystallized and then converted into the acid potassium salt, the salt of saccharic acid was obtained.

The analytical results obtained on anhydrosaccharic acid were as follows:

0.1000 gm. substance: 0.0348 gm. K_2SO_4.
$C_6H_7O_7Ka+H_2O$.   Calculated.   K 15.70.
                                         Found.   " 15.61.

The optical rotation of the substance was:

$$[\alpha]_D^{20} = \frac{+ 0.63° \times 100}{1 \times 1} = + 63°$$

The analytical results obtained on the salt of the saccharic acid were as follows:

0.1000 gm. substance: 0.0352 gm. K_2SO_4.

$$C_5H_9O_8K. \quad \text{Calculated.} \quad K \; 15.70.$$
$$\text{Found.} \quad \text{``} \quad 15.80.$$

The optical rotation of the substance is as follows:

$$[\alpha]_D^{20} = \frac{+\,0.07° \times 100}{1 \times 1} = +\,7°$$

Benzylidene-1-Ethyl-2-Bromomannonate.—The diazo compound (20.0 gm.) is suspended in 400.0 cc. of anhydrous (over sodium) ether and dry ether saturated with hydrogen bromide gas is added as long as the diazo compound is dissolved and the yellow color has disappeared. The ethereal solution is then washed with a saturated solution of sodium carbonate, cooled to 0°C. The operation is repeated until all hydrobromic acid is removed from the ethereal solution. The ethereal solution is then dried with anhydrous sodium sulfate, concentrated to a small volume, and the bromo compound is precipitated with ligroin (80-90°C.). The crude compound is obtained analytically pure after several recrystallizations, from a mixture of ether and ligroin. The yield was between 25 to 40 gm. from 100.0 gm. of the diazo compound. The substance melted at 119°C. (corrected) and analyzed as follows:

0.1040 gm. substance: 0.1820 gm. CO_2 and 0.0480 gm. H_2O.
0.2000 " " : 0.1016 gm. AgBr.

$$C_{15}H_{19}BrO_6. \quad \text{Calculated.} \quad C \; 47.99, \; H \; 5.10, \; B \; 21.30.$$
$$\text{Found.} \quad \text{``} \; 47.25, \; \text{``} \; 5.16, \; \text{``} \; 21.56.$$

The optical rotation of the substance was:

$$[\alpha]_D^{20} = \frac{-\,0.66° \times 100}{1 \times 2} = -\,33°$$

Benzylidene-1-Ethyl-2,3-Anhydromannonate.—The substance was obtained in an experiment which aimed to convert the bromo compound into the corresponding amino derivative.

The bromo compound (30.0 gm.) is dissolved in 35 cc. of 98.5 per cent alcohol and poured into 30.0 cc. of concentrated aqueous ammonia. After standing for 5 to 10 minutes at room temperature the substance solidifies on cooling into a solid mass consisting of long microscopic needles. The material is filtered, dried in a vacuum desiccator over sulfuric acid and then recrystallized

from 35 per cent alcohol. This operation is repeated several times. Finally the product is twice recrystallized in its own weight of boiling 98.5 per cent alcohol. The final product melted at 122.5°C. (corrected) and analyzed as follows:

0.1130 gm. substance: 0.2522 gm. CO_2 and 0.0594 gm. H_2O.
$C_{15}H_{18}O_6$.   Calculated.   C 61.21, H 6.12.
Found.   " 60.86, " 5.88.

The optical rotation of the substance was:

$$[\alpha]_D^{20} = \frac{-1.10° \times 100}{1 \times 1.5} = -73.3°$$

Benzylidene-Ethyl-Desoxyglueonate (*Mannonate*).—The previous substance (93.0 gm.) was dissolved in 10 cc. of 98.5 per cent alcohol and saturated with hydrogen gas in the presence of Paal's colloidal palladium. The substance absorbed the theoretical volume of hydrogen. The operation was completed in 72 hours. The product was filtered. The greatest part of the palladium, however, remained in colloidal solution. Hence the filtrate was concentrated to dryness under diminished pressure. The residue was dissolved in a little boiling alcohol with charcoal, filtered, and allowed to evaporate to dryness. This operation was repeated three times when a perfectly colorless product was obtained. It melted at 126°C. (corrected) and analyzed as follows:

0.1081 gm. substance: 0.2274 gm. CO_2 and 0.0642 gm. H_2O.
$C_{15}H_{20}O_6$.   Calculated.   C 60.81, H 6.76.
Found.   " 60.91, " 7.05.

The optical rotation of the substance was:

$$[\alpha]_D^{20} = \frac{-0.52° \times 100}{1 \times 2} = -26°$$

Benzylidene-1-Amino-2,3-Anhydrogluconate (*Mannonate*).—When a solution of the bromo compound in alcohol is added to ammonia water following exactly the same conditions as for the preparation of the benzylidene-ethyl-anhydrogluconate, and is allowed to stand at 0°C., the solution remains either liquid or partly gelatinous. On further cooling in an ice-alcohol mixture the contents of the flask turn into a gelatinous mass. This is best filtered and washed with water, it then acquires a white granular character. After it is dried in a vacuum desiccator (over soda-lime) to complete dryness it can be recrystallized from absolute alcohol. The

process may be repeated until the product is analytically pure. The substance melted at 230°C. and analyzed as follows:

0.1106 gm. substance: 0.2380 gm. CO_2 and 0.0586 gm. H_2O.
0.0993 " " required (Kjeldahl) 3.64 cc. 0.1 N acid.
$C_{13}H_{15}N O_5$. Calculated. C 58.84, H 5.70, N 5.28.
Found. " 58.68, " 5.93, " 5.14.

The substance had the following rotation:

$$[\alpha]_D^{20} = \frac{+ 1.30° \times 100}{1 \times 2} = + 65°$$

Action of Aqueous Ammonia on Benzylidene-2-Bromo-Ethyl-Gluconate.—Benzylidene-2-bromo-ethyl-gluconate (5.0 gm.) was suspended in 100 cc. of 2 per cent sulfuric acid and placed on a water bath for 1 hour. The benzaldehyde settled out on the bottom of the flask in the form of an oil. The oil was extracted by means of ether and the aqueous solution freed from sulfuric acid quantitatively by means of barium hydroxide. The aqueous solution was then concentrated to a volume of 20 cc., an equal volume of concentrated ammonia water was added, and the solution was kept in a sealed tube at 100°C. for 24 hours. The resulting solution after the removal of the ammonia, showed the presence only of a few mm. of amino nitrogen.

Benzylidene-1-Ethyl-2-Chlorogluconate.—The substance was prepared in the same manner as the corresponding bromo compound, with the exception that ether saturated with hydrogen chloride gas was used. The yield was 10.0 gm. from 100.0 gm. of the diazo compound. It melted at 127°C. and analyzed as follows:

0.1062 gm. substance: 0.2122 gm. CO_2 and 0.0584 gm. H_2O.
0.2024 " " : 0.0858 " AgCl.
$C_{15}H_{19}Cl O_6$. Calculated. C 54.46, H 5.8, Cl 10.74.
Found. " 54.69, " 6.11, " 10.48.

The rotation of the substance was:

$$[\alpha]_D^{20} = \frac{- 0.20° \times 100}{1 \times 1} = - 20°$$

Benzylidene-1-Amino-2-Chloromannonate.—The substance described in the previous section (5.0 gm.) was dissolved in 5 cc. of 98.5 per cent alcohol and transferred into 4.0 cc. of aqueous ammonia. The solution was allowed to stand over night and then concentrated in a vacuum desiccator. A deposit, consisting of microscopic needles, was formed. This was recrystallized

first from 35 per cent alcohol and finally from 98.5 per cent alcohol until the substance gave a negative test with Nessler's reagent. The substances melted at 197°C. (corrected) and analyzed as follows:

0.1022 gm. substance: 0.1938 gm. CO_2 and 0.0540 gm. H_2O.
0.1857 " " : 0.0844 " AgCl.
0.1887 " " required (Kjeldahl) 5.95 cc. 0.1 N acid.
  $C_{13}H_{16}NO_5Cl$. Calculated. C 51.73, H 5.35, N 4.69, Cl 11.75.
    Found. " 51.72, " 5.52, " 4.41, " 11.23.

The substance had the following rotation:

$$[\alpha]_D^{20} = \frac{-0.23° \times 100}{1 \times 1} = -23°$$

Conversion of the Benzylidene-1-Ethyl-2-Chloromannonate into Chitosaminic Acid.—The chloro compound (3.0 gm.) was dissolved in 4 cc. of 98.5 per cent alcohol and the solution added to 4.0 cc. of aqueous ammonia. This is sealed in a tube and heated at 95°C. for 10 hours. At the end of that time the tube is allowed to cool and the solution which has then turned dark brown is concentrated to nearly dryness under diminished pressure. The residue is taken up in 2 per cent sulfuric acid and boiled over a flame for 15 minutes. It is then allowed to cool and washed with ether, (in a separatory funnel) to remove the benzaldehyde. The resulting aqueous solution is freed from hydrochloric acid and ammonia in the usual way and concentrated to nearly dryness. The residue is dissolved in a little water, acetone is added to the solution until an oil settles out, and all is warmed on a water bath until crystallization begins. Prior to treatment with acetone a small sample of the aqueous solution was used for an amino nitrogen determination according to Van Slyke. On the basis of this estimation the solution contained 0.9 gm. of chitosaminic acid. However, from five experiments only 2.5 gm. of chitosaminic acid crystallized. After one recrystallization the substance was analytically pure. It analyzed as follows:

0.1108 gm. substance: 0.1508 gm. CO_2 and 0.0674 gm. H_2O.
0.0670 " " required (Kjeldahl) 3.45 cc. 0.1 N acid.
    $C_6H_{13}NO_6$. Calculated. C 36.92, H 6.66, N 7.18.
      Found. " 37.11, " 6.80, " 7.21.

The rotation of the substance was:

$$[\alpha]_D^{20} = \frac{-0.15° \times 100}{1 \times 1} = -15°$$

THE FATE OF SOME OF THE PHENYLACETYLATED AMINO-ACIDS IN THE ANIMAL ORGANISM.

By GEORGE J. SHIPLE and CARL P. SHERWIN.

(From the Chemical Research Laboratory of Fordham University, New York.)

Magnus-Levy endeavored (1) to account for the formation of the large quantities of glycocoll which the body can furnish after the ingestion of benzoic acid on the theory that hippuric acid might possibly originate by the curtailment, as it were, of longer chained benzoylated amino-acids. Accordingly, he benzoylated ten of the known amino-acids and injected the compounds subcutaneously into dogs. Quantitative analysis of the urine showed that in no case was there any evident increase in the amount of hippuric acid excreted. On the contrary, the investigator was able to recover the original substances from the urine, and this in such quantities that the absence of any such partial demolition of the longer chained amino-acids was assured. Ando (2) took α-aminocinnamic acid, which is known to be completely oxidized in the body, and benzoylated it. He found that both after ingestion and injection, it was eliminated in the urine unchanged. When, however, benzoylated tyrosine or benzoylated p-hydroxy-α-aminocinnamic acid was fed, these compounds were almost entirely destroyed in the organism.

It would seem therefore, that an aliphatic α-amino-acid, regardless of the length of its chain, is not subject to oxidation as long as the α-amino group remains intact, and that the same applies also to the aromatic amino-acids, unless as in the cases of tyrosine and p-hydroxy-α-aminocinnamic acid, the para position of the ring has been subjected to previous partial oxidation. Moreover, the evidence seems conclusive that the molecule in the above cases was rendered impervious to the attempts at oxidative or hydrolytic deamination by the replacement of one of the hydrogen atoms of the α-amino group by a benzoyl radical. Further-

463

more, as other investigators have shown, the same successful "blocking" of the α-amino group has been effected by the formation of the uramino compound or of the hydantoin. Thus Salkowski (3) showed that when hydantoic acid is fed to rabbits it is excreted as such in the urine; and Rolfde (4) isolated the greater part of the uramino derivative of leucine from the urine of a cat after the intravenous injection of the substance. Finally, Lewis and Root (5) have recently shown that the phenyluramino derivative of cystine probably passes through the body without undergoing oxidation, for there is little increase in the amount of oxidized sulfur in the urine after the substance has been fed, and no increase in this form of sulfur after the injection of the substance. For further corroboration of this work the reader is referred to the very recent article of Hijikata (6) who has described in detail the "rediscovery" of some of the facts mentioned above.

In our work we had in mind four different problems: (a) the preparation and study of a number of the phenylacetyl derivatives of the natural amino-acids; (b) the determination of the efficiency of this phenylacetyl radical as a "block" to the catabolism of these amino-acids; (c) an attempt to solve the mystery regarding the genesis of glycocoll in the animal organism; and (d) a study of the physiological effects produced on one animal by the detoxication products elaborated by an animal of another species.

Accordingly, we prepared the phenylacetyl derivatives of glycocoll, alanine, leucine, glutamine, glutamic acid, asparagine, aspartic acid, and ornithine, according to the Schotten and Baumann method, namely by shaking a weakly alkaline solution of the amino-acid with phenylacetyl chloride. Of these compounds, phenylacetyl alanine and phenylacetyl leucine had never been prepared before. They were studied, therefore, in somewhat greater detail.

The phenylacetyl radical, differing chemically but little from its homologue, the benzoyl radical, was expected to act as a complete "block" of the amino group, and therefore to furnish little additional evidence or information in this respect above that supplied by the results obtained by Magnus-Levy. In one way, however, it seemed to offer a new and interesting clue to the solution of

the mystery.   Benzoic acid combines with glycocoll in the human organism as well as in that of the lower animals and is excreted in the urine of both as hippuric acid.   Phenylacetic acid, however, combines in the human body with the longer chained amino-acid glutamine, and is excreted as phenylacetyl glutamine or phenyl-acetyl glutamine urea, but in the bodies of the lower animals it simply combines with glycocoll and appears in the urine as phenaceturic acid.   It seemed that this phenylacetyl glutamine in the brute organism might possibly be an intermediary product in the detoxication of phenylacetic acid, which product is then subjected to further decomposition before elimination, with the formation of phenaceturic acid.   In other words, the splitting off of three carbon atoms from the glutamine molecule would result in the production of glycocoll.   This idea was further strengthened by the fact which was found later that glutamine as well as glyco-coll can be made synthetically by the human body at the expense of nitrogen which otherwise would have appeared in the urea fraction (7).   Thus a man placed on a carbohydrate diet and reduced to a condition of endogenous protein catabolism was fed phenylacetic acid.   It was found that his urea nitrogen dropped from about 75 to 28 per cent of the total nitrogen.   After feeding benzoic acid much the same thing occurred.   The drop in urea nitrogen in the latter case, though not so marked, was still suffi-cient (from about 75 to about 50 per cent of the total nitrogen) to show that a substantial part of the urea nitrogen had been used for the synthesis of glycocoll.   It is particularly interesting to note here that benzoic acid and phenylacetic acid may be detoxi-cated simultaneously as easily as either of the two singly, or in other words, both glycocoll and glutamine can be made by the body simultaneously as easily as when but one is demanded.

Phenylacetic acid (8) like benzoic acid (9) is detoxicated in the organism of the fowl by a combination of 2 molecules of the acid with 1 molecule of ornithine.   The analogy between the two com-pounds, glutamine and ornithine, is too striking to need further comment.

We wished especially to determine the physiological behavior of these detoxication products of phenylacetic acid in organisms other than those in which the original detoxication occurred, for it seemed that these products would either be remodelled into the

detoxication product of phenylacetic acid common to that species of animal or be still further changed chemically in order to reduce the toxicity and facilitate the rapid elimination of the resulting substance in the urine. Phenylacetyl glutamine was therefore prepared and fed as well as injected into dogs, cats, and chickens. Phenylacetyl ornithine (phenacetornithuric acid) was prepared and fed to dogs and to human beings; and phenylacetyl glycocoll (phenaceturic acid) was prepared and fed to human beings as well as to chickens.

(Phenylacetyl glycocoll)   (Phenylacetyl glutamine)   (Phenylacetyl ornithine)

Nearly every protein has yielded on hydrolysis certain quantities of glutamic acid and with this an amount of ammonium salts proportionate to the glutamic acid. Accordingly, the inference has been drawn that glutamic acid exists in the protein molecule in the form of the amide, scl. as glutamine, which has been known for some time to exist in plant proteins. We, therefore, prepared phenylacetyl glutamic acid both from phenylacetyl glutamine as well as by the Schotten and Baumann synthesis from phenylacetyl

chloride and *d*-glutamic acid. This compound was then fed to human beings in order to determine whether the phenylacetyl glutamine found in the urine of a man after the ingestion of phenylacetic acid could be a secondary product formed from phenylacetyl glutamic acid. The phenylacetyl glutamic acid was also fed to rabbits and to chickens. The phenylacetyl derivatives of asparagine, aspartic acid, alanine, and leucine were fed to dogs, chickens, and to human beings to determine what effect they would have regarding the formation of the detoxication products of phenylacetic acid common to these species.

When a human being was used as the subject in these experiments the substances were ingested as a water solution of the sodium salt. In the cases of the lower animals, including the chickens, the solution of the sodium salt was fed by means of a stomach tube and washed down with plenty of water. When the substances were injected, the exactly neutral sodium salt was prepared and dissolved in isotonic salt solution. When the lower animals were employed as subjects of experimentation, for example dogs, cats, and rabbits, they were placed in metabolism cages. The urine was then collected for a suitable period of time (12, 24, or 36 hours) depending upon the amount of the material ingested. The urine was evaporated to a thick syrup, acidified to Congo red with dilute sulfuric acid, and extracted with a suitable solvent (alcohol, ether, or ethyl acetate) in a rotary extractor of a modified Richter type. The compounds were sometimes crystallized directly from the organic solvent, as in the case of the phenylacetyl glutamine, from the ethyl acetate. In most cases, however, the organic solvent was evaporated to dryness, the residue taken up with water, decolorized with animal charcoal, and recrystallized several times from hot water.

The work on chickens was very much hindered by the great difficulty of separating the urine from the feces. The birds were placed in metabolism cages large enough to allow the free movement of the animal. The most satisfactory flooring material for the cage was found to be a stiff wire net of about $\frac{1}{2}$ inch mesh. This allowed free passage of the urine and the feces into a removeable drawer in the bottom of the cage. The feces and the urine were thus collected together, dried in a current of warm air if necessary, then mixed in a flask with five to ten times their weight

of alcohol. The flask, with frequent shaking, was then allowed
to stand for at least about 40 hours. After this time the contents
were filtered and the alcoholic solution was evaporated to dryness
in vacuo or in a current of warm air. The residue was then ex-
tracted with a small volume of ether to get rid of the fats which
are ever present in the alcoholic extract. The mass was next
taken up with a small amount of water, acidified to Congo red
with dilute hydrochloric acid, and extracted repeatedly with the
organic solvent which was known to be suitable for the suspected
compound (ether or ethyl acetate was generally used). In the
preparation of the dibenzoyl ornithine (ornithuric acid) it was
found most profitable simply to place the ether extract in the ice
box for from 1 to 3 weeks and wait until the substance crystallized
out. In the preparation of the diphenylacetyl ornithine, however,
we found that the process might be hastened by evaporating the
ether solution to dryness, then taking the residue up in alcohol
(in which the material is very soluble), and gradually diluting the
alcoholic solution with water. The phenacetornithuric acid is
thus forced out of solution in the form of needles when the dilu-
tion is made carefully, and as an amorphous powder when the
water is added in too large amounts.

EXPERIMENTAL.

1. *Phenylacetyl Glycocoll.*

Phenylacetyl glycocoll (phenaceturic acid) was prepared ac-
cording to the Schotten and Baumann reaction by adding to a
glycocoll solution small portions (about $\frac{1}{3}$ cc.) of phenylacetyl
chloride together with sufficient sodium hydroxide to keep the
mixture faintly alkaline. In the meantime the container was
constantly shaken. The best results were obtained when not less
than 2 molecules of the acid chloride were used for each molecule
of glycocoll. After all the acid chloride was added the material
was acidified to Congo red with dilute hydrochloric acid and
extracted three times with ether to remove the phenylacetic acid
which was formed during the operation. The phenaceturic acid
was usually present then as a flocculent, white precipitate. This
was then filtered and recrystallized three times from hot water,
after which it was found to be relatively pure. It melted at 142–

143°C., and according to the Kjeldahl method contained 7.18 per cent nitrogen instead of the theoretical 7.25 per cent.

The substance was weighed out in 5 gm. doses and dissolved in hot water. Sodium hydroxide was then added until neutrality was reached. The solution of the sodium salt thus formed was drunk by a man of 65 kilos body weight who was chosen as the subject of experimentation. The material had no particularly bad taste and proved to be entirely non-toxic. In fact its toxicity was so low that 2 days later the same subject ingested 10 gm. in the course of 24 hours (5 gm. in the morning and 5 gm. in the afternoon of the same day). After a dose of 5 gm., 3.72 gm. of the phenaceturic acid were recovered from the urine, and after a dose of 10 gm., 7.62 gm. were found in the urine. No free phenylacetic acid could be found nor any conjugation product of the same other than the glycocoll compound.

A hen weighing 1.75 kilos was fed on 3 consecutive days 0.5 gm., 1 gm., and 2 gm., respectively, of phenaceturic acid as a solution of the sodium salt by means of a stomach tube. No signs of intoxication appeared. The excreta were treated according to the general plan described above. Of the 3.5 gm. fed, 2.6 gm. of the material were recovered unchanged, but no trace of a compound of ornithine with phenylacetic acid could be found.

2. Diphenylacetyl Ornithine (Phenacetornithuric Acid).

This substance was obtained according to the method of Totani (9) from the excreta of hens after they had been fed phenylacetic acid. The material, though somewhat more easily prepared than its homologue, dibenzoyl ornithine (8), required several weeks to obtain enough for feeding experiments. In our work (10) we have since found that the easiest way to prepare either the benzoyl or the phenylacetyl derivative of ornithine is to place the chickens on a carbohydrate diet for a short time and feed them 1 or 2 gm. of benzoic acid or phenylacetic acid per day. Not only are the hens able to build the ornithine compound very well on a non-protein diet, contrary to the statements found in the literature, but the feces under these conditions are reduced to a minimum and the quantity of urine is increased.

1 gm. of the diphenylacetyl ornithine, in the form of a solution of the sodium salt, was fed to a small dog of 2.7 kilos body weight.

In the urine was found a small amount of phenaceturic acid. No phenylacetyl ornithine, however, could be recovered, probably due, in part at least, to both the small amount of the material ingested as compared with the large amount of solid matter in the urine, as well as to the extreme solubility of the sodium salt of the phenacetornithuric acid. It seemed quite probable, moreover, that the formation of the phenaceturic acid in the organism of the dog was not due to the metabolic alteration of the ornithine into glycocoll, but rather to the splitting of the quite unstable ornithine compound into phenylacetic acid and ornithine, followed by a conjugation of the phenylacetic acid with glycocoll. Accordingly, 1 gm. of the phenacetornithuric acid was converted into the sodium salt, dissolved in isotonic salt solution, and injected subcutaneously into a rabbit. From the urine of the rabbit we were able to recover about 0.2 gm. of phenylacetyl ornithine. Not a trace, however, of the phenaceturic acid could be found. Apparently, therefore, when the compound was not subjected to the digestive processes of the gastrointestinal tract, there was no hydrolysis of the material into its components and consequently no alteration of the compound in the processes of metabolism.

A man ingested two doses of 2 gm. each of phenacetornithuric acid, only the lack of material limiting the size of the dose, for the substance proved to be utterly non-toxic. After the first ingestion of 2 gm. the urine was collected for a period of 12 hours only, evaporated to a thick syrup, acidified, and extracted for 2 hours with absolute ethyl acetate. No crystals of phenylacetyl glutamine appeared on cooling nor on concentration of the extract. It is safe to say, therefore, that no phenylacetyl glutamine was formed. After the ethyl acetate had been evaporated to dryness and the residue had been dissolved in a large volume of ether and allowed to stand in the cold for some time, a very small amount of phenacetornithuric acid (0.1 gm.) crystallized out. After a dose of 2 gm. of the phenacetornithuric acid with a subsequent 24 hour collection of urine, neither phenylacetyl glutamine nor phenylacetyl ornithine could be obtained from the evaporated urine. It is safe to say, however, that the failure to obtain the latter was due to the large amount of solid matter in the residue after so long a urine collection.

3. *Phenylacetyl Glutamine.*

This substance was prepared according to the method of Thierfelder and Sherwin (11). A man of 62.5 kilos body weight ingested 5 gm. of phenylacetic acid on each of 3 consecutive days. The urine was evaporated to a thick syrup, acidified, and extracted with ethyl acetate. From the ethyl acetate extract was obtained a mixture of phenylacetyl glutamine and phenylacetyl glutamine urea. The mixture of the two substances was then taken up in a saturated solution of barium hydroxide and allowed to stand for a number of hours. In this way the urea was split off from the phenylacetyl glutamine and the barium salt of the latter formed. After standing in the ice box for about 12 hours this solution was treated with carbon dioxide to remove the excess barium. The neutral solution was then evaporated to dryness *in vacuo,* the residue extracted with hot absolute alcohol to remove the urea, the barium salt of the phenylacetyl glutamine dissolved in a small amount of water and acidified with sulfuric acid to remove the barium as barium sulfate, the water solution of the phenylacetyl glutamine extracted with absolute ethyl acetate and the pure phenylacetyl glutamine obtained. That this compound was identical with that originally obtained by Thierfelder and Sherwin is shown by its constants. After drying *in vacuo* at 70°C. the substance melted at 101–104°C., and according to the Kjeldahl method contained 10.49 per cent nitrogen instead of the theoretical 10.69 per cent. The substance was also levorotary.

Phenylacetyl glutamine was fed to a dog in 3 gm. doses as a solution of the sodium salt. A dog of 15 kilos body weight thus received two 3 gm. doses at 12 hour intervals. The urine was collected for 24 hours after the last dose, and from it we obtained 2 gm. of phenylacetyl glutamine and 0.4 gm. of phenaceturic acid. In order to avoid a splitting of the phenylacetyl glutamine in the gastrointestinal tract, the substance was next injected subcutaneously in 1 gm. doses as the sodium salt in isotonic salt solution at 3 hour intervals until 4 gm. in all had been administered. From the subsequent urine we recovered 2.1 gm. of phenylacetyl glutamine (M.P. 99–102°C.), but found no trace of phenaceturic acid.

Phenylacetyl glutamine was fed to a hen in 1 gm. doses as a solution of the sodium salt. The hen received in this way 3 gm.

of the substance in the course of 2 days. The excreta were collected, dried, extracted with alcohol, the alcoholic solution was evaporated to dryness *in vacuo*, taken up with water, acidified to Congo red with dilute hydrochloric acid, and extracted with ethyl acetate to remove the phenylacetyl glutamine. After the separation of the phenylacetyl glutamine from the ethyl acetate, the latter was evaporated to dryness *in vacuo* and the residue extracted repeatedly with ether to remove any diphenylacetyl ornithine. After the feeding of the 3 gm. to the hen, only 0.6 gm. of the original phenylacetyl glutamine was recovered, but no diphenylacetyl ornithine could be found.

4. *Phenylacetyl d-Glutamic Acid.*

This material was prepared according to the method of Schotten and Baumann by shaking phenylacetyl chloride in excess with a weakly alkaline solution of *d*-glutamic acid (M.P. 203°C.). The same compound was also prepared from phenylacetyl *d*-glutamine by boiling the latter with a saturated solution of barium hydroxide under a reflux condenser until there were no more fumes of ammonia evolved. As this compound of phenylacetic acid is a thick syrup which can be crystallized only with great difficulty, its water solution was employed in all the feeding experiments.

A human being ingested 5 gm. of the substance; 1.5 gm. were fed to a chicken, and 2 gm. to a rabbit. From the urine of the human being 3.2 gm. of the compound were isolated unchanged. From the excreta of the hen 0.45 gm. of the original substance was obtained, and 1.1 gm. of it were recovered from the urine of the rabbit. In no case, however, was any other phenylacetic acid conjugate obtainable.

5. *Phenylacetyl Asparagine.*

This compound was prepared, like the preceding ones, according to the Schotten and Baumann process. It crystallizes easily, although the yield is very poor. It was identical with the substance previously prepared (12) as shown by the melting point of 180–181°C. after drying at 80°C. *in vacuo*. It contained according to the Kjeldahl method 11.09 per cent nitrogen instead of the theoretical 11.20 per cent.

Phenylacetyl asparagine is apparently somewhat more toxic than the other phenylacetyl derivatives of the amino-acids. A man of 62 kilos body weight ingested 6 gm. of the substance in 2 gm. doses at 24 hour intervals. Each dose was followed by a general feeling of depression and later by severe frontal headaches. From the urine 3.8 gm. of the material were recovered unchanged. After feeding 1 gm. of the compound to a hen in 0.25 gm. doses, 0.2 gm. of it was recovered unchanged, and after the administering of 1 gm. to a rabbit in the same manner, 0.6 gm. of it was recovered from the urine.

6. *Phenylacetyl Alanine.*

This compound, which had never been studied before, was synthesized as follows: 10 gm. of inactive alanine were dissolved in 50 cc. of water and shaken with about 50 gm. of phenylacetyl chloride (3 molecules of phenylacetyl chloride to 1 molecule of alanine). The acid chloride was added in small amounts with continuous shaking. Sodium hydroxide solution was added in quantities just sufficient to keep the mixture alkaline. The entire process lasted about 2 hours. During the operation it was necessary to stop occasionally and cool the container in ice water to remove the heat of reaction. After the last of the acid chloride had been added, the contents of the bottle were transferred to a 1 liter separatory funnel and acidified to Congo red with dilute sulfuric acid. There appeared at once a heavy, milky mass of solid material. The mixture was then extracted several times with benzene to remove the phenylacetic acid which had formed during the reaction. The white substance which remained after the extraction with benzene, proved by analysis (see below) to be phenylacetyl alanine. The yield from 10 gm. of alanine was about 70 per cent (16 gm.). The compound is fairly soluble in cold water and very soluble in hot water. From the water solution it crystallizes in snow-white, feathery clusters, which when dried at 80°C. melt at 150–152°C. It is quite soluble in ether, ethyl acetate, alcohol, carbon tetrachloride, and *hot* benzene. In the form of a saturated water solution the material was optically inactive. The sodium salt, which is very soluble in water, was found to be also optically inactive. When dried at

80°C. *in vacuo*, the compound showed the following analytical results:

Analysis of Phenylacetyl Alanine.

	Calculated.	Found.
	per cent	*per cent*
C	63.78	63.77
H	6.33	6.34
O	23.17	23.07
N	6.76	6.34

To form the barium salt of the phenylacetyl alanine, 0.75 gm. of the substance was dissolved in a cold, saturated solution of barium hydroxide. Carbon dioxide was then passed through the solution to remove the excess barium. The barium salt was found to be extremely soluble in cold water, in fact, so soluble that it refused to crystallize even after the solution had been evaporated to a thick syrup.

In order to split the inactive material into its optically active isomers, compounds of the phenylacetyl alanine with brucine, strychnine, quinine, and cinchonine were formed, but all of them were so extremely soluble that it was impossible to obtain any of them in the crystalline form.

We fed 3 gm. of the phenylacetyl alanine in the form of a water solution of the sodium salt to a small dog. The material was apparently physiologically inactive for the animal ate and drank as usual immediately after the feeding. The 24 hour collection of urine was evaporated to a thick syrup. Upon acidification it became almost solid due to the separation of the phenylacetyl alanine. The material was then transferred to a separatory funnel, extracted twice with ethyl acetate, the ethyl acetate evaporated to dryness, and the residue taken up with water from which it was recrystallized. The crystals thus obtained melted sharply at 151–153°C., showing that the phenylacetyl alanine had passed through the organism unchanged, and this almost quantitatively, for of the original 3 gm. that had been fed 2.75 gm. were recovered.

A hen was fed 3 gm. of phenylacetyl alanine in 1 gm. doses on 3 consecutive days. The material was found to be entirely non-

toxic. From the excreta only 1 gm. of the original substance was recovered, but no ornithine compound of phenylacetic acid could be obtained. The substance which was isolated melted at 148–150°C. A human being after ingesting 4 gm. of the phenylacetyl alanine noticed no ill effects. From the urine about 2.5 gm. of the material could be recovered.

7. *Phenylacetyl dl-Leucine.*

This compound was prepared by dissolving 3 gm. of inactive leucine in 60 cc. of water, placing the solution in a 250 cc. bottle, then adding phenylacetyl chloride in small portions (about 10 gm. in all) with constant shaking, keeping the mixture alkaline the while by the addition of small amounts of sodium hydroxide solution. The entire operation lasted about 2 hours. At the end of this time the contents of the bottle were poured into a 500 cc. separatory funnel and acidified to Congo red with dilute sulfuric acid. Thereupon a mass of white material was thrown out of solution. The acidified mixture was extracted several times with carbon tetrachloride to remove the free phenylacetic acid. The white, flocculent material remaining was then filtered by suction and recrystallized several times from water. When dried at 80°C. *in vacuo* for several hours it melted at 133–134°C. Analysis (see below) proved it to be phenylacetyl leucine. The compound is a white material, crystallizing from hot water in beautiful, feathery clusters. It is very soluble in alcohol, ether, ethyl acetate, and acetone; moderately soluble in benzene; slightly soluble in cold water; but much more easily in hot water. It is just about absolutely insoluble in carbon tetrachloride and petroleum ether. The yield of this and similar syntheses was only about 55 per cent of the theoretical. On analysis, after drying *in vacuo* at 80°C., the following results were obtained:

Analysis of Phenylacetyl Leucine.

	Calculated.	Found.
	per cent	per cent
C...	67.44	67.49
H...	7.68	7.80
O...	19.26	18.99
N...	5.62	5.72

The sodium, potassium, and ammonium salts of this compound are too soluble to be obtained in the crystalline form. The barium salt is soluble in 25 parts of water and crystallizes in regular and well defined bundles of needles.

The physiological behavior of the phenylacetyl leucine was much the same as that of the other phenylacetylated amino-acids. It was injected intravenously into a rabbit in a dose as large as 1 gm. without evident physiological effects. The original phenylacetyl leucine was recovered afterwards from the urine in an amount which was about 65 per cent of the quantity injected. When 1 gm. of the phenylacetyl leucine was fed to a chicken, the same results were obtained, for from the excreta 0.67 gm. of the substance was recovered. A dose of 4 gm. was taken by a man without causing discomfort. From the evaporated urine 2.27 gm. of the original material were isolated. Apparently it had gone through the organism as the very soluble sodium salt.

DISCUSSION.

The amino group of the α-amino-acids is "blocked" as effectively by the phenylacetyl radical as by the benzoyl radical. In only one instance was there an apparent chemical reaction involving one of the phenylacetylated amino-acids; namely, when diphenyl-acetyl ornithine was fed to a dog. Here there was evidently an hydrolysis of the compound in the gastrointestinal tract into phenylacetic acid and ornithine, for there was found a small amount of phenaceturic acid in the urine after the feeding but none after the intravenous injection of the material.

It would seem that there is no support to the still extant theory concerning the partial oxidation of benzoylated amino-acids into hippuric acid, nor is there any reason to believe that the amino-acid can enter into chemical reaction of any kind so long as one of the hydrogen atoms of the amino group is replaced by some other radical or element.

At the present time all the evidence seems to indicate that certain amino-acids, namely glycocoll, glutamine, ornithine, and perhaps even cystine, can be built by the animal organism when the subject is in a condition of endogenous protein catabolism, that is, when every avenue to outside protein material or nitrogen of every kind is closed. Furthermore, we know that the nitrogen

for the amino group is obtained from that portion of nitrogen which would otherwise appear in the urea fraction. The problem still confronts us, however, as to the manner in which the amino-acids are catabolized in the animal organism. Are all the different amino-acids normally burned completely into ammonia, carbon dioxide, and water, and is the glycocoll then resynthesized from these end-products, or is glycocoll a common product of the intermediary metabolism of all of these amino-acids? This latter seems rather improbable, since glutamine which contains both an amino and an amide group is synthesized as easily as is glycocoll, and certainly glutamine cannot be considered an intermediary product in the oxidation of any except a very few of the amino-acids at most.

It seems peculiar, to say the least, that there should be apparently three entirely different detoxication processes going on in the organisms of the dog, the hen, and the human being, respectively, when each of the resulting compounds is perfectly harmless when taken into the organism of the other, from which it is easily and rapidly eliminated by way of the urine. At the present time there is no evident explanation of this apparent incongruity. Further investigation of this matter is necessary.

Berczeller (13) has suggested, in connection with the detoxication of foreign organic compounds, that the more important change to be kept in mind is not the chemical alteration of the toxic substance, but rather a change of a purely physical nature. He cites several cases of toxic compounds and compares them with their respective detoxication products. Invariably he finds that there is decidedly a less pronounced lowering of the surface tension of the solution by the detoxication product than by the toxic substance itself. This effect he considers to be the chief aim of the body during the detoxication process. Thus benzoic acid lowers the surface tension of the solution very markedly, while hippuric acid produces a very slight effect in that regard. Again, menthol causes a very decided lessening of the surface tension, while the glycuronic acid compound of menthol has much less effect, and the sodium salt of the latter has practically no such effect at all. Finally, phenol sulfuric acid has only about one-tenth the reducing effect on the surface tension of the solution as has phenol itself. This may be the explanation of the phenom-

enon, it is true, but still undiscovered is the mechanism of the synthetic method by which these amino-acids are prepared in the animal body.

SUMMARY.

Phenylacetyl derivatives of the following natural amino-acids were prepared: glycocoll, alanine, leucine, glutamine, glutamic acid, asparagine, aspartic acid, and ornithine. Of these compounds, phenylacetyl alanine and phenylacetyl leucine were studied in greater detail since their preparation and constants had not been recorded in the literature.

All the above substances were fed to or injected into dogs, rabbits, chickens, and human beings. In every case results showed that when the amino group is phenylacetylated, complete or even partial catabolism of the amino-acid is prevented, thus demonstrating the impossibility of the formation of glycocoll from a more complex amino-acid under these conditions.

Most peculiar and interesting, moreover, is the fact that although different species detoxicate phenylacetic acid according to entirely different reactions which yield completely different compounds, still these products pass unaltered through the organisms of animals other than those in which the original detoxication occurred.

BIBLIOGRAPHY.

1. Magnus-Levy, A., *Biochem. Z.*, 1907, vi, 555.
2. Ando, H., *J. Biol. Chem.*, 1919, xxxviii, 7.
3. Salkowski, E., *Z. physiol. Chem.*, 1880, iv, 100.
4. Rohde, A., *J. Biol. Chem.*, 1918, xxxvi, 467.
5. Lewis, H. B., and Root, L. E., *J. Biol. Chem.*, 1922, l, 303.
6. Hijikata, Y., *J. Biol. Chem.*, 1922, li, 141.
7. Shiple, G. J., and Sherwin, C. P., *J. Am. Chem. Soc.*, 1922, xliv, 618.
8. Jaffe, M., *Ber. chem. Ges.*, 1877, x, 1925.
9. Totani, G., *Z. physiol. Chem.*, 1910, lxviii, 75.
10. Crowdle, J. H., and Sherwin, C. P., Unpublished results.
11. Thierfelder, H., and Sherwin, C. P., *Ber. chem. Ges.*, 1914, xlvii, 2630.
12. Thierfelder, H., and Sherwin, C. P., *Z. physiol. Chem.*, 1915, xciv, 1.
13. Berczeller, L., *Biochem. Z.*, 1917, lxxxiv, 75.

THE RELATION BETWEEN AGE AND THE CONCENTRATIONS OF PROTEIN FRACTIONS IN THE BLOOD OF THE CALF AND COW.

By PAUL E. HOWE.

(From the Department of Animal Pathology of The Rockefeller Institute for Medical Research, Princeton, N. J.)

The marked variations in the quantities of certain protein fractions present in the blood of the new-born calf (1, 2) and the differences between the blood of young calves and of the adult cow led us to study the changes which occur in bovine blood with increasing age. For this purpose we have studied the blood of three calves from birth to an age of approximately 2 to 3 months. Samples of blood have been taken from other animals; three heifers 6 months old, three heifers 12 months old, fifteen virgin heifers 17 to 22 months old, and fourteen pregnant heifers $2\frac{1}{2}$ years old. The heifers 17 to 22 months old were about to be bred and those 30 months old had been pregnant about 3 months. These observations are supplemented by analyses of the blood of other animals for a short period (2) and data obtained in studies in another connection.

Of the calves which were studied continuously one (Calf 669) received colostrum of a high protein content, a second (Calf 889) received colostrum which was comparatively poor in globulin, and a third (Calf 898) did not receive colostrum but was fed ordinary whole milk from birth. Blood was collected by needle from the jugular vein and when plasma was obtained coagulation was prevented by sodium citrate. Blood samples were taken 1 hour after feeding. The calves were fed ordinary whole milk for 1 month after which they were given small amounts of grain and hay.

Determinations were made of total nitrogen, fibrin, or fibrinogen nitrogen, nitrogen content of the protein precipitated by con-

Protein Fractions in Blood

centrations of sodium sulfate of 14.2, 17.4, and 21.5 per cent, and of the non-protein nitrogen. From these determinations values were calculated for fibrin, euglobulin, pseudoglobulins I and II, and albumin, according to the procedures previously

TABLE I

Data Relating to the Variations in the Blood Proteins of Calf 898 Which Did Not Receive Colostrum soon after Birth. *

Age.	Total N.	"Serum" N.	Fibrin N	Euglobulin N	Pseudoglobulin I N	Pseudoglobulin II N.	Total globulin exclusive of fibrin	Albumin N.	Non-protein N
New-born.	0.636	0.581	0.055	0.016	0.028	0.144	0.189	0.322	(0.070)
days									
1	0.659	0.602	0.057	0.008	0.050	0.144	0.202	0.330	(0.070)
2	0.703	0.604	0.099	0.002	0.052	0.139	0.183	0.351	(0.070)
3	0.758	0.636	0.122	0.000	0.046	0.159	0.205	0.361	(0.070)
4	0.757	0.594	0.163	0.045	0.025	0.120	0.190	0.359	0.045
6	0.862	0.656	0.206	0.047	0.046	0.157	0.240	0.379	0.037
8	0.901	0.668	0.233	0.008	0.060	0.126	0.194	0.437	0.037
14	0.957	0.763	0.194	0.023	0.051	0.124	0.198	0.511	0.054
20	0.887	0.759	0.138	0.029	0.012	0.153	0.194	0.511	0.054
27	0.854	0.740	0.114	0.037	0.049	0.107	0.183	0.516	0.041
34	1.024	0.804	0.220	0.048	0.069	0.149	0.260	0.503	0.041
41	0.977	0.876	0.101	0.035	0.120	0.111	0.266	0.560	0.050
48	0.974	0.944	0.030	0.095	0.101	0.113	0.309	0.577	0.058
55	0.961	0.887	0.074	0.037	0.132	0.103	0.272	0.570	0.045
62	0.998	0.935	0.063	0.072	0.113	0.165	0.350	0.541	0.045
69	0.895	0.841	0.054	0.049	0.095	0.099	0.243	0.553	0.045
76	0.891	0.847	0.054	0.051	0.099	0.074	0.224	0.590	0.033
83	0.875	0.828	0.047	0.052	0.104	0.084	0.240	0.548	0.040
90	0.918	0.841	0.077	0.063	0.100	0.088	0.261	0.544	0.036
97	0.959	0.862	0.097	0.066	0.088	0.136	0.290	0.532	0.040
111	0.981	0.908	0.073	0.049	0.114	0.124	0.287	0.573	0.048

* Results are expressed as grams of nitrogen in 100 cc. of blood plasma or serum.

outlined (3). The data are contained in Tables I to VI and Chart 1. The relation between the corpuscles and plasma was determined by measuring the volume of each after centrifuging at a constant speed. The average proportions of corpuscles and plasma for Calves 898 and 899 were 38 to 62 and 44 to 55, respectively. These relations held throughout the experiment.

In the chart the data from each of the calves (Calves 669,[1] 899, 898), have been plotted for each of the blood constituents estimated with the exception of the total plasma nitrogen and the non-protein nitrogen. In place of the total plasma nitrogen,

TABLE II.

Data Relating to the Variations in the Blood Proteins of Calf 899 Which Received Colostrum Relatively Low in Globulin. *

Age.	Total N.	"Serum" N.	Fibrin N.	Euglobulin N.	Pseudoglobulin I N.	Pseudoglobulin II N.	Total globulin exclusive of fibrin.	Albumin N.	Non-protein N.
Colostrum.	1.916	Casein=1.122		0.258	0.181	0.095		0.101	0.062
New-born.	0.685	0.623	0.062	0.033		0.186	0.219	0.363	0.041
days									
1	0.877	0.764	0.093	0.116	0.204	0.136	0.451	0.276	0.038
2	0.949	0.808	0.141	0.128	0.151	0.145	0.424	0.335	0.049
3	0.950	0.853	0.097	0.115	0.185	0.174	0.474	0.334	0.045
4	1.006	0.866	0.140	0.132	0.198	0.144	0.474	0.347	0.048
5	0.848	0.726	0.122	0.073	0.146	0.166	0.375	0.306	0.045
8	0.927	0.792	0.135	0.083	0.144	0.132	0.359	0.392	0.041
12	0.935	0.852	0.083	0.136	0.141	0.152	0.429	0.367	0.054
18	0.878	0.794	0.084	0.072	0.124	0.103	0.299	0.450	0.045
25	0.901	0.833	0.068	0.078	0.093	0.140	0.311	0.483	0.041
32	0.947	0.883	0.064	0.095	0.083	0.165	0.343	0.495	0.045
39	0.882	0.824	0.058	0.043	0.083	0.100	0.226	0.548	0.050
46	0.955	0.899	0.056	0 072	0.087	0.142	0.301	0.540	0.058
53	0.864	0.827	0.037	0.054	0.100	0.133	0.287	0.482	0.058
60	0.923	0.842	0.081	0.075	0.093	0.097	0.265	0.532	0.045
67	0.856	0.784	0.072	0.050	0.087	0.127	0.264	0.475	0.045
68	0.858	0.796	0.062	0.083	0.084	0.104	0.272	0.487	0.037

* Results are expressed as grams of nitrogen in 100 cc. of blood plasma or serum.

values for serum nitrogen obtained from the analysis of the filtrates from the precipitation of fibrin or fibrinogen have been recorded. These results were taken for comparison because the fibrinogen of blood appears to vary somewhat independently of the other blood constituents.

[1] Data relating to the absorption and disappearance of agglutinins of Calf 669 have been presented in another connection (4).

From an inspection of the tables and of the chart the following points are evident:

Serum Nitrogen.—During the first weeks of life the quantity of serum nitrogen present in the blood of young calves depends

TABLE III.

Data Relating to the Variations in the Blood Proteins of Calf 669 Which Received Colostrum Relatively High in Globulin.*

Age.	Total N.	"Serum" N.	Fibrin N.	Euglobulin N.	Pseudoglobulin I N.	Pseudoglobulin II N.	Total globulin exclusive of fibrin.	Albumin N.	Non-protein N.
New-born.		0.768		0.039	0.051	0.175	0.265	0.422	(0.081)
2hrs. 40 mins.		0.768		0.000	0.128	0.177	0.282	0.405	(0.081)
5 " 40 "		0.960		0.133	0.264	0.162	0.559	0.319	0.081
19 " 40 "		1.126		0.307	0.333	0.158	0.798	0.290	0.038
days									
3		1.160		0.298	0.320	0.133	0.751	0.358	0.051
13		1.186		0.299	0.141	0.111	0.551	0.558	0.077
21		1.071		0.239	0.128	0.073	0.440	0.597	0.034
31		0.972		0.064	0.174	0.069	0.307	0.622	0.043
41		1.005		0.045	0.196	0.071	0.312	0.667	0.026
50		0.994		0.062	0.115	0.115	0.294	0.666	0.034
57		0.959		0.051	0.098	0.102	0.251	0.661	0.047
65		0.937		0.033	0.189	0.077	0.289	0.601	0.047
71		0.948		0.082	0.120	0.098	0.289	0.616	0.043
78		0.917		0.030	0.106	0.107	0.233	0.653	0.021
85		0.937		0.042	0.058	0.087	0.187	0.713	0.037
92		0.931		0.036	0.111	0.085	0.232	0.658	0.041
106		1.032		0.071	0.093	0.173	0.337	0.646	0.049
113		0.959		0.072	0.091	0.153	0.319	0.584	0.049
118		1.038		0.081	0.114	0.113	0.308	0.681	0.049

* Results are expressed as grams of nitrogen in 100 cc. of blood plasma or serum.

upon the quantitative nature of the diet just after birth. If the calf does not receive colostrum but is fed ordinary milk the serum nitrogen increases gradually for about 6 weeks. This increase is due, essentially, to the increase in albumin. In case colostrum has been ingested the serum nitrogen values indicate the effect of the absorption of globulins from the colostrum. After approxi-

TABLE IV.

Data Relating to the Proteins in the Blood of Calves 6 and 12 Months Old.

Age.	Total N.	"Serum" N.	Fibrin N.	Euglobulin N.	Pseudoglobulin I N.	Pseudoglobulin II N.	Total globulin exclusive of fibrin.	Albumin N.	Non-protein N.
mos.									
	1.098	1.027	0.071	0.124	0.210	0.132	0.466	0.516	0.045
6	1.082	0.991	0.091	0.104	0.223	0.120	0.447	0.494	0.050
	1.030	1.002	0.028	0.129	0.178	0.140	0.437	0.507	0.058
Average....	1.070	1.007	0.063	0.116	0.203	0.131	0.450	0.506	0.051
	1.087	1.038	0.049	0.077	0.206	0.136	0.419	0.547	0.062
12	1.074	1.006	0.068	0.070	0.210	0.145	0.425	0.523	0.058
	1.059	1.021	0.028	0.097	0.210	0.161	0.468	0.503	0.050
Average....	1.073	1.022	0.048	0.081	0.208	0.137	0.437	0.524	0.057

TABLE V.

*Data Relating to the Proteins in the Blood of Different Non-Pregnant Heifers 18 to 22 Months Old.**

Age.	Total N.	"Serum" N.	Fibrin N.	Euglobulin N.	Pseudoglobulin I N.	Pseudoglobulin II N.	Total globulin exclusive of fibrin.	Albumin N.	Non-protein N.
mos.									
17	1.132	1.027	0.105	0.083	0.202	0.156	0.441	0.516	0.070
18	1.219	1.043	0.176	0.115	0.260	0.120	0.495	0.478	0.070
18	1.359	1.270	0.089	0.181	0.355	0.126	0.672	0.520	0.078
18	1.104	1.002	0.102	0.049	0.256	0.103	0.408	0.524	0.070
18	1.060	0.978	0.082	0.034	0.235	0.091	0.363	0.545	0.070
19	1.185	1.130	0.055	0.070	0.256	0.152	0.478	0.582	0.070
19	1.082	1.002	0.080	0.062	0.231	0.136	0.429	0.515	0.058
20	1.099	1.047	0.052	0.090	0.239	0.145	0.474	0.515	0.058
20	1.305	1.126	0.179	0.042	0.395	0.157	0.594	0.462	0.070
20	1.261	1.134	0.127	0.057	0.380	0.132	0.569	0.511	0.054
20	1.316	1.114	0.202	0.042	0.375	0.140	0.557	0.499	0.058
21	1.175	1.035	0.140	0.078	0.279	0.138	0.495	0.490	0.050
22	1.168	1.097	0.071	0.035	0.353	0.132	0.520	0.515	0.062
22	1.183	1.048	0.135	0.058	0.301	0.136	0.495	0.483	0.070
22	1.184	1.084	0.100	0.036	0.293	0.136	0.464	0.557	0.062
Average...	1.188	1.073	0.115	0.069	0.296	0.133	0.494	0.514	0.064

* Results are expressed as grams of nitrogen in 100 cc. of blood plasma or serum.

mately 6 weeks serum nitrogen fluctuates somewhat but is lower than the concentration found in the adult animal or in the calves 6 months old. The adult values appear to be attained between 3 and 6 months of age.

Albumin Nitrogen.—Changes in the albumin nitrogen are more or less independent of the variations in the other proteins. The

TABLE VI.

Data Relating to the Proteins in the Blood of Different Pregnant Heifers 30 Months Old.

Age.	Total N.	"Serum" N.	Fibrin N.	Euglobulin N.	Pseudoglobulin I N.	Pseudoglobulin II N.	Total globulin exclusive of fibrin.	Albumin N.	Non-protein N.
mos.									
	1.079	1.015	0.064	0 099	0.260	0.112	0.471	0.482	0.062
	1.052	0.964	0.088	0.061	0.214	0.136	0.411	0.491	0.062
	1.142	1.083	0.059	0.072	0.293	0.128	0.493	0.528	0.062
	1.043	0.957	0.086	0.116	0.251	0.136	0.503	0.396	0.058
	1.129	1.070	0.059	0.101	0.268	0.120	0.489	0.523	0.058
	1.085	1.033	0.052	0.072	0.297	0.148	0.517	0.458	0.058
30	1.064	1.010	0.054	0.129	0.198	0.099	0.416	0.532	0.062
	1.095	1.047	0.048	0.086	0.243	0.120	0.449	0.540	0.058
	1.225	1.136	0.089	0.092	0.364	0.140	0.596	0.478	0.062
	1.123	1.085	0.038	0.194	0.256	0.132	0.582	0.441	0.062
	1.133	1.074	0.059	0.158	0 207	0.144	0.509	0.499	0.066
	1.090	1.021	0.069	0.130	0.177	0.161	0 468	0.487	0.066
	1.150	1.070	0.080	0.196	0.288	0.096	0.580	0.423	0.066
	1.163	1.113	0.050	0.189	0.285	0.136	0.610	0.437	(0.066)
Average....	1.112	1.048	0.064	0.121	0.252	0.132	0.506	0.479	0.062

value at birth is slightly higher than on the following day. From this time there is a gradual increase up to approximately 3 weeks, after which there is a tendency toward a slight increase with irregular fluctuations. The important fact to be brought out with regard to the albumin nitrogen is that changes in the concentration of albumin, particularly in the first 3 weeks of life, do not appear to be correlated with the changes in the concentration of the globulins.

Fibrin Nitrogen.—The data on fibrin nitrogen are in part, in the case of Calves 898 and 899 and of the older animals, based on duplicate determinations by two different procedures; coagulation following recalcification with calcium chloride and precipitation with 10.6 per cent sodium sulfate. The first procedure represents fibrin and the second fibrinogen. With the quantities of plasma used the results by the two methods usually agree.[2]

From the data on Calves 898 and 899 it might appear that there is a higher fibrinogen content of the blood early in life, during the period when adjustments in the other proteins are taking place. The determinations of fibrin on various young animals indicate a considerable individual variation and no relation of age to the fibrinogen concentration of the blood. A consideration of data on the adult animals also tends to substantiate this idea. It is evident that the variations in the concentration of fibrinogen of the calves is not directly related to the variations in the other serum proteins. The daily records on Calves 898 and 899 do not lend much assistance in interpreting the variations in the fibrinogen content of the blood. Calf 898 had a temperature approximately 1° higher than Calf 899 at the time the fibrinogen content of its blood was rising but during the period of high fibrinogen values the temperature was approximately that of Calf 898. From the work of Smith and Little (5) on the effect of colostrum upon the new-born animal, it is probable that this calf was conducting a battle against an infection which was not present in the case of Calf 899. The large increase in fibrinogen at the age of 34 days in the case of Calf 898 occurred at the same time as an abraised and swollen knee-joint which was not present the period before this one and had disappeared before the time of the next analysis. Calf 899 does not present any marked temperature changes. In the latter part of the experiment he was subject to a deranged digestive apparatus due to the ingestion of binding twine in his bedding which caused partial occlusion of the rumen and interfered with his normal metabolic activities. The data obtained during the last month of life for this calf are open to question as far as it may be considered as representing a normal animal.

[2] Unpublished data.

CHART 1.

486

CHART 1. Curves showing the variations in various protein fractions of blood with increasing age. Calf 898 did not receive colostrum, Calf 899 received colostrum relatively low in globulins, and Calf 669 received colostrum rich in globulins. The crosses (✕) indicate results obtained on calves which did not receive colostrum; some of these animals were injected with cow serum, some were injected and fed cow serum, and some were fed cow serum (5). The results on these animals agree essentially with those obtained on animals which had not received colostrum or which would have received milk containing very small amounts of globulins. The plus signs (+) indicate results on animals which had received colostrum soon after birth. Average data for calves 6 and 12 months of age, non-pregnant heifers 17 to 22 months old, and pregnant heifers 30 months old are plotted as indicated in the legend. The data are presented in terms of grams of nitrogen in 100 cc. of blood plasma or serum.

The work of Foster and Whipple (6) indicates that cell injury, inflammation, intoxication, or liver injury, will affect the formation of fibrin. If we admit the probable systemic infection (5), then the general high fibrin values for Calf 898 and the increase in fibrin at the time of the swollen knee-joint might be explained by the findings of these investigators.

Total Globulin.—In the case of Calf 898 which did not receive colostrum the values for total globulin nitrogen remain practically constant for 4 weeks after which they increase to values which are essentially the same as those for calves which received colostrum. At about 10 weeks there appears to be a tendency for the total globulin to decrease slightly. At 6 months the total globulin values approach those of the adult animal. The two calves which received colostrum showed a marked increase in globulin during the first day of life after which the total globulin shows a tendency to decrease during a period of from 3 to 4 weeks. Following this change the amount of total globulins is approximately the same regardless of the previous diet. The concentration of globulin present during the first 3 to 4 weeks of life is directly related to the quantity of globulin absorbed during the first days of life. Colostrum ingested by Calf 669 was very thick and rich in globulin whereas the colostrum ingested by Calf 899 was relatively poor in globulin.

Euglobulin.—Remarks with regard to the ingestion of colostrum and the variation in protein nitrogen relating to the total globulin apply to euglobulin. At birth there is essentially no euglobulin present. When euglobulin is not obtained from the colostrum or otherwise it appears in the blood gradually and at approximately 5 to 6 weeks reaches the values found in calves which received colostrum. The euglobulin content of the adult animal is variable, due to influences which have not been determined. The data relating to euglobulin in the case of Calf 898 show a certain quantity of euglobulin during the first week of life. These values are probably only in a degree accurate and are due in part to technical error. The reason for this assumption is, that in the case of serum at such ages there is not a visible precipitation at 14.2 per cent of sodium sulfate.

Pseudoglobulin I.—In the first weeks of life the pseudoglobulin 1 content of the blood is related to the nature of the diet im-

mediately after birth. At an age of approximately 5 to 6 weeks the values are the same no matter what the diet may have been. The adult animal has a much higher pseudoglobulin I content than the calf 3 months old.

Pseudoglobulin II.—The quantity of protein precipitated, between 17.4 and 21.5 per cent of sodium sulfate, appears to be independent of the diet and is practically constant for all ages. This condition exists in spite of considerable fluctuations in the protein content of the blood on either side of this fraction.

Animals 6 to 30 Months of Age.—Data relating to the concentration of the various protein fractions in animals 6 to 30 months of age are contained in Tables IV to VI. The average values are indicated in Chart 1. The blood samples for calves 6 and 12 months old were collected at the same time and under similar feeding conditions. The samples from the virgin heifers and the pregnant heifers were collected about 2 weeks apart. The general conditions of stabling and of feeding were essentially the same; the animals were still on winter feed and had not been pastured. The results of the analyses indicate certain minor individual variations in the distribution of the proteins of the blood. The effect of age is not particularly apparent after the calves are 6 months old. The only indication of a difference between the two groups of adult animals is in the euglobulin fraction which is higher in the non-pregnant heifers than in the pregnant heifers. On the other hand, the concentrations of fibrin are in general higher in the virgin heifers than in the pregnant heifers. This is rather surprising since, from the work of Fahræus (7) on human serum there is a greater suspension stability in pregnant than in non-pregnant women. The suspension stability appears to be in part at least related to the fibrinogen and globulin fractions of the plasma, in which fibrinogen has a greater individual effect than the other globulins. Our observations do not necessarily contradict those of Fahræus for we are dealing with a different organism and we have not made determinations of suspension stability.

A calculation of the relative proportions of globulin nitrogen and albumin nitrogen to the total nitrogen gives for virgin heifers 49 per cent of the total serum protein as globulin and 51 per cent as albumin; for the pregnant heifers the values are 51 per cent of

total globulin nitrogen and 49 per cent of albumin nitrogen. These values differ from those of Robertson (8, 9) who found 36 per cent of total globulins and 64 per cent of total albumins for the ox. Robertson's average values for Hammarsten's (10) determinations of ox serum are 58 per cent total globulin and 42 per cent total albumin. We have found a number of cases in which the serum of the adult animal contained a preponderance of globulin over the albumin but only a few animals, except in the case of calves, in which the albumins predominated over the globulins. We are dealing, of course, with the cow while Robertson may have been studying the steer; we do not have any evidence relating to sex.

<div style="text-align:center">DISCUSSION.</div>

Studies of the variations in the distribution of proteins with increasing age are comparatively few. The most detailed investigations are those of C. E. Wells (11) on the rabbit and of Toyama (12) on the albino rat. Toyama's investigation followed that of Hatai (13) on the total protein content of the serum of the same animal. Reiss (14) and Utheim (15) have made observations on infants. These investigators have all determined the proteins by means of the refractometer. In the work on rabbits and rats the procedure of Robertson (9) for the separation of proteins has been used. Lewis and H. G. Wells (16) have recently presented some analyses of human blood using the method employed in this work.

It is necessary to be very cautious in comparing results obtained upon different species of animals. The work of Robertson (9) has brought out species, as well as individual, differences in the proportions of albumin and globulin in the serum of the rat, rabbit, horse, and ox. A difference between the infant and the calf is shown in the data of Lewis and Wells. These investigators confirm to a certain extent for the infant our observations on calves; they found that blood obtained from the umbilical cord of infants does not contain euglobulin. On the other hand, their data indicate quantities of pseudoglobulin I in the blood of infants comparable to those present in adult man, whereas in calves this protein is essentially absent.

The most extended series of analyses of the total protein of the blood of infants and children has been made by Utheim (15) who confirms the work of Reiss. It was found that the concentration of total protein remains practically constant at 6 to 6.5 per cent from birth to about the 10th to 11th month when it begins to rise. The adult level is reached at about the 15th month. The serum of premature infants contains less protein, 4.5 per cent, than that of infants born at full term. The normal level for infants is not attained until about 3 months of age.

Alder (17) has studied the blood of man by means of the refractometer and viscosimeter and finds practically no difference between men and women; little difference in the composition of blood serum (a) between the ages of 7 and 70 years, (b) as a result of the ingestion of food, (c) following muscular activity, (d) between venous and capillary blood, and (e) from day to day. Placental blood contained, as a rule, less protein and a higher proportion of albumin than adult blood. The percentage of total protein in placental blood, 5.7 to 7.0 per cent, is higher than that for new-born calves, 3.6 to 5.6 per cent. Most of the values for calves lie between 3.6 and 4.8 per cent; only two samples out of twenty-eight showed a value above 4.8 per cent, the average is 4.4 per cent. The results of Lewis and Wells on placental blood, 4.3 to 6.7 per cent (N $\times$ 6.25), agree in general with those of Alder.

From the consideration of published data it is apparent that the blood of a new-born animal has a lower total protein content than that of the adult animal and that during the early part of life there is an increase in the total protein concentration of the serum. In infants and rats the protein content of the serum the first days after birth appears to be slightly lower than at birth or a few days later. With rats (13) there is a rapid increase in protein up to the time of weaning at which time the protein content shows some irregularity. The increase then continues until sexual maturity is attained when there is again an irregularity followed by further slight increases to the adult level. In the case of rabbits (11) and infants (13, 14) the available data indicate a gradual increase in total protein from birth to maturity.

In the studies just reviewed the effect of the nature of the diet immediately following birth has not been considered. The data

presented in this paper indicate that the quantity of total protein present in the serum of calves is definitely related to the quantitative composition of the colostrum or milk ingested soon after birth. The *quantitative* variations in the composition of blood plasma imposed by the absorption of the proteins of colostrum are transient. At the age of from 4 to 6 weeks, the quantitative effect of the absorbed protein has practically disappeared and the composition in the blood serum tends to become the same no matter what the previous diet may have been. These remarks apply to the normal animal.

The relative distribution of the various protein fractions of blood serum with increasing age has been studied extensively in but two cases, Wells on rabbits and Toyama on white rats. Wells did not find any "correspondence between the ages of the animals and the variations of the relative proportions of 'insoluble' globulin, 'soluble' globulin and albumins" in the blood serum of the rabbit. His youngest animal was 21 days old. The data of Toyama relate to the rat from birth to maturity. He found a gradual increase in quantity of globulin and albumin present in the blood serum, which was most rapid in the suckling period, 23 days. Immediately following the suckling period, at 30 days, there was a fall in the quantity of globulin but not in the albumin, while at the next age studied, 50 days, the albumin values dropped slightly while the globulin showed an increase.

The data presented on calves and cows indicate that during approximately 4 to 6 weeks of life the proportions of the different protein fractions precipitated from the blood by sodium sulfate are affected by the diet of the calf soon after birth; *i.e.*, by the character of the milk ingested. Following this period the absolute and relative proportions of globulins are approximately the same. The proportions of globulins characteristic of the adult animal are attained at the age of from 18 to 22 months. When colostrum containing euglobulin and pseudoglobulin I is fed there is a rapid absorption of protein by the calf (1). The absorbed globulins then gradually disappear, in part at least. When globulins are not absorbed they are formed gradually and attain values similar to those which occur following the ingestion of colostrum at about the time when the absorbed globulins have fallen to the average values for a calf 4 to 6 weeks old. The variations in globulin content just

discussed relate particularly to those globulins precipitated by concentrations of sodium sulfate less than 17.4 per cent, euglobulin and pseudoglobulin I. Pseudoglobulin II remains relatively constant at all times. This constancy of the pseudoglobulin II fraction suggests that the variations in the other proteins with feeding and age are true variations in protein and not the secondary effect of changes in the water content of the blood; *i.e.*, that the water content is adjusted to changes in the protein content of the blood.

The albumin concentration of calf serum is low at birth and rises rather rapidly during the first 2 weeks, at the end of which time the adult level is approached. Variations in the concentrations of albumin are apparently not affected by the ingestion of colostrum; essentially the same conditions exist whether or not globulin is absorbed. The non-protein nitrogen appears to be higher at birth, then to decrease slightly, and to rise again to the adult value, which approaches that at birth.

The proportions of albumin and globulin in non-pregnant and pregnant heifers are approximately equal. This statement applies to separations made with sodium sulfate. We have reason to believe that similar results would be obtained with ammonium sulfate and possibly a slightly higher proportion of globulin would be indicated when magnesium sulfate is used as the total globulin precipitant. The most marked difference between the adult blood and that of a calf 3 months old is in the proportion of pseudoglobulin I present in the blood. It appears that one of the chief adjustments between these ages is in the pseudoglobulin I fraction. The data which we have presented as indicating the composition of adult blood apply to females which have just reached maturity. Certain analyses indicate that the blood of older animals may vary widely, particularly in the fibrinogen, euglobulin, and pseudoglobulin I fractions. Some animals have a relatively high euglobulin concentration.

BIBLIOGRAPHY.

1. Howe, P. E., *J. Biol. Chem.*, 1921, xlix, 115.
2. Orcutt, M. L., and Howe, P. E., *J. Exp. Med.*, 1922, xxxvi, in press; *Proc. Soc. Exp. Biol. and Med.*, 1921–22, xix, 331.
3. Howe, P. E., *J. Biol. Chem.*, 1921, xlix, 109.

4. Little, R. B., and Orcutt, M. L., *J. Exp. Med.*, 1922, xxxv, 161.

5. Smith, T., and Little, R. B., *J. Exp. Med.*, 1922, xxxvi, in press.

6. Foster, D. P., and Whipple, G. H., *Am. J. Physiol.*, 1921–22, lviii, 407.

7. Fahraeus, R., *Acta Med. Scand.*, 1921, lv, 3.

8. Robertson, T. B., *J. Biol. Chem.*, 1912, xi, 179.

9. Robertson, T. B., *J. Biol. Chem.*, 1912–13, xiii, 325.

10. Hammarsten, O., *Arch. ges. Physiol.*, 1878, xvii, 413.

11. Wells, C. E., *J. Biol. Chem.*, 1913, xv, 37.

12. Toyama, I., *J. Biol. Chem.*, 1919, xxxviii, 161.

13. Hatai, S., *J. Biol. Chem.*, 1918, xxxv, 527.

14. Reiss, E., *Jahrb. Kinderh.*, 1909, lxx, 311.

15. Utheim, K., *Am. J. Dis. Child.*, 1920, xx, 366.

16. Lewis, J. H., and Wells, H. G., *J. Am. Med. Assn.*, 1922, lxxviii, 863.

17. Alder, A., *Deutsch. Arch. klin. Med.*, 1918, cxxvi, 61.

THE RATE OF HYDROLYSIS OF WHEAT GLIADIN.*

By HUBERT BRADFORD VICKERY.

(From the Laboratory of the Connecticut Agricultural Experiment Station, New Haven.)

Braconnot in 1820 was the first to hydrolyze proteins with acid. He boiled meat as well as glue with sulfuric acid and isolated leucine and glycocoll from the resulting mixtures. These were the first amino-acids obtained from protein substances.

Alkalies were also used as hydrolyzing agents at an early date, for Mulder (1839) obtained leucine from meat which had been boiled with sodium hydroxide.

The view that proteins are complex compounds built up of amino-acids originated with Liebig, but the manner in which amino-acids are linked with each other in the protein molecule has only been established in recent years. In 1902 Hofmeister thoroughly examined and discussed the various ways in which one might imagine two amino-acids could be united with each other in the protein molecule, and pointed out that the only linkage which was entirely probable was that which involved the scheme,

$$R - NH - CO - R$$

This linkage has been termed by Fischer the peptide bond. The actual existence of this configuration in the protein molecule has been established by the work of Fischer and Abderhalden and their associates, who have isolated numerous polypeptides from partially hydrolyzed proteins. But

---

whether or not all the amino-acids are united in peptide union in the protein molecule, is a question that cannot be answered by work of this nature.

The peptide bond is split on hydrolysis with the formation of an amino group and a carboxyl group. Consequently, if the peptide bond occurs extensively in the protein molecule, the products of hydrolysis should contain large amounts of free carboxyl and amino groups. The isolation of much of the nitrogen of proteins in the form of amino groups of amino-acids renders it highly probable that the amino-acids actually are united in the protein molecule in peptide union, but proof of this can only be obtained when the progress of hydrolysis has been shown to be accompanied by the formation of free amino and carboxyl groups.

It is obvious that a study of the progress of hydrolysis of proteins is dependent upon the development of an analytical method whereby either free amino or free carboxyl groups may be estimated in the hydrolysis mixture. The earliest purely chemical method for doing this is due to Siegfried (1905) who found that amino-acids would unite in the cold with calcium hydroxide and carbon dioxide to form calcium salts of carbamino-acids. On heating, these decompose and an amount of calcium carbonate precipitates which is equivalent to the amount of amino nitrogen present. Mathieu (1909) made use of this property to follow the rate of hydrolysis of gelatin by boiling 1.55 M acid, but without very satisfactory results.

Sörensen in 1908 introduced the formol titration method whereby the amount of carboxyl liberated by the hydrolysis of peptide bonds could be measured. This method was used by Henriques and Gjaldbäk in 1910 to ascertain the conditions under which proteins could be hydrolyzed completely, and in 1911 to follow the enzymatic hydrolysis of several proteins. It is evident from their results that after the protein is completely hydrolyzed the solution contains large amounts of free amino and carboxyl groups and also, that the process of hydrolysis actually is accompanied by the formation of increasing amounts of these two groups. Although Henriques and Gjaldbäk apparently did not appreciate it themselves, this was the first definite chemical evidence that the amino-acids were for the most part united in the protein molecule by means of the peptide bond.

Van Slyke in 1911 introduced his nitrous acid method to estimate free amino nitrogen and the application of this method to the products of protein hydrolysis by numerous investigators has served further to verify the views of Hofmeister and Fischer.

Nasse in 1872 clearly pointed out that the nitrogen which gives rise to ammonia on hydrolysis of proteins must be bound differently in the molecule from the nitrogen which is found as amino groups of amino-acids after hydrolysis. Since that time Nasse's "loosely bound nitrogen" has been variously termed "amide nitrogen" or "ammonia nitrogen," but no very clear evidence that the ammonia was derived from an amide group in the protein molecule was presented until 1908, when Osborne, Leavenworth, and Brautlecht pointed out the close correspondence between the amount of ammonia required by theory for amide formation with one of the carboxyl groups of the aspartic and glutaminic acids in numerous proteins, and the

amount of ammonia actually obtained from them after acid hydrolysis. Further evidence was obtained by Thierfelder and von Cramm (1919) who found the proportions of ammonia removed by a definite mild hydrolysis from synthetic polypeptides containing glutamine to be almost identical with the proportion removed from gliadin under the same conditions. Furthermore, Osborne and Nolan (1920) demonstrated the appearance of an acidity in the solution when gliadin was hydrolyzed by dilute acid, which was closely equivalent to the amount of ammonia liberated by the hydrolysis, and which was best explained by the hypothesis that the ammonia was derived from amides of the dicarboxylic acids.

Dakin's (1918) discovery of oxyglutaminic acid, which he found in casein, gliadin, and glutenin, and which was found by Jones and Johns (1921) in lactalbumin, has raised a question as to the value of conclusions regarding the origin of ammonia from amide nitrogen based upon the amounts of glutaminic and aspartic acids obtained from proteins. Nevertheless, it seems almost certain that the large amounts of ammonia obtained from most proteins must be derived from the hydrolysis of amides of these dicarboxylic acids.

The discovery of the basic amino-acids, of tryptophane, and of proline, in proteins has shown that in addition to the simple peptide bond and the grouping which gives rise to the ammonia, nitrogen occurs in a guanidino group (arginine), in an imidazole group (histidine), and in an indole ring (tryptophane) as well as in the pyrollidine ring of proline and oxyproline. Moreover, the small amount of free amino nitrogen found only in those proteins which contain lysine is probably due to the end-standing amino group of this amino-acid. These nitrogenous systems are all, save tryptophane, stable to acid, but the guanidino group of arginine is quite readily decomposed by alkalies, breaking down to form carbon dioxide and ammonia. Tryptophane is also unstable to alkalies.

When methods involving the measurement of amino nitrogen are used to determine the rate at which a protein is hydrolyzed, account must be taken of the observation of Fischer and Abderhalden (1904), that proline can enter into polypeptide union not only with its carboxyl group, but also with its imino nitrogen group. A union of this latter type if existing in a protein would be hydrolyzed without the formation of an amino group and would hence escape detection. There is no method at present available to detect imino nitrogen peptide union if it occurs in the protein molecule.

In addition to the types of union which have been definitely proved to occur in proteins there are other types which possibly may exist in them. Andersen and Roed-Müller (1915) have presented evidence which indicates the possible presence of very small amounts of uramino-acids, although substances containing the uramino group or derivatives of it, have never been isolated from proteins. Johnson and Burnham (1911) have drawn attention to the possibility that sulfur may occur in proteins in thiopeptide, $-CS - NH -$, union which implies the existence of nitrogen in a grouping different from any of those mentioned.

From these considerations it is apparent that a study of the rate of hydrolysis of proteins is limited to the study of the rate at which, on the one hand, ammonia appears in the solution, and on the other hand, the rate at which peptide bonds are broken with the formation of amino groups. Such a study shows only the rate of decomposition of the hypothetical amide group and the rate of hydrolysis of the peptide bonds, exclusive of those in which the imino group of proline plays a part.

The rate at which proteins are hyrolyzed by *acids or alkalies* has received very little study since the introduction of suitable analytical methods, although much attention has been paid to the rate of hydrolysis by enzymes.

Van Slyke (1911) made use of his nitrous acid method to estimate amino nitrogen to study the rate of hydrolysis of egg albumin by 5 per cent sodium hydroxide at 60°, but his experiments were carried out mainly with the object of demonstrating the applicability of the method.

Pittom in 1914 used Sörensen's formol titration to follow the rate of hydrolysis of casein and egg albumin when boiled with 20 per cent hydrochloric acid. He also determined the rate at which ammonia is set free from these proteins as well as the rate at which substances precipitable by phosphotungstic acid are hydrolyzed, and showed that each of these reactions has a very high velocity in the early stages of the hydrolysis. The ammonia was practically all set free within an hour while the amino nitrogen rapidly increased in amount during the first 2 or 3 hours, the rate of hydrolysis thereafter being much slower.

Harding and Fort (1918) followed the rate of hydrolysis of the protein of the human placenta by boiling 20 per cent hydrochloric acid, using Van Slyke's method.

The rate of hydrolysis of proteins by enzymes has been studied by Henriques and Gjaldbäk (1911) using Sörensen's method; Walters (1912, *a* and *b*), who worked with casein and precipitated the unaltered casein by neutralization and then estimated the nitrogen in the precipitate; Andersen (1915) also using Sörensen's method, who determined how far hydrolysis could be carried by enzymes alone; and by Frankel (1916), Northrop (1919), and Dunn and Lewis (1921) who followed the rate of enzymatic hydrolysis of numerous proteins by means of Van Slyke's method.

We have studied the rate at which ammonia is set free from gliadin by various concentrations of hydrochloric acid ranging from 0.1 to 20 per cent at boiling temperature, at the same time observing the rate of hydrolysis of the peptide bonds as indicated by the appearance of free amino nitrogen in the solution. In addition we have carried out a few series of experiments with sulfuric acid, as well as with sodium and barium hydroxides, in order to ascertain the effect upon the rate of hydrolysis of a less highly ionized acid and of alkaline reagents.

Gliadin was selected for our experiments since it can be readily obtained in a state of purity, it has been thoroughly investigated with regard to its amino-acid make-up, and moreover, it contains a larger proportion of amide nitrogen, glutaminic acid, and proline than any other protein hitherto analyzed.

Osborne and Nolan (1920) observed that 1 per cent hydrochloric acid liberates almost as much ammonia from gliadin on boiling for 2 hours as is set free by 20 per cent hydrochloric acid on boiling for 24 hours. We have found that ammonia is set free from gliadin quite rapidly at first by boiling 0.027 N (0.1 per cent) hydrochloric acid. Under the experimental conditions adopted, the neutralization of the acid by the ammonia set free is, in this case, the deciding factor in the rate of hydrolysis. Nevertheless, hydrolysis slowly proceeded until when the solution had been boiled for 118 hours, over 90 per cent of the free acid had been thus removed.

By boiling with 0.1 N hydrochloric acid nearly all the ammonia is set free from gliadin in 11 hours, while with 0.2 N acid the same point is reached in 5 hours. The rate of liberation of ammonia by acids more concentrated than 1.0 N is so rapid in the early stages as to render measurement impossible by the methods used, but with these higher concentrations of acid another phenomenon becomes evident. It has long been known that the total amount of ammonia obtainable from a protein could be slightly increased by prolonged hydrolysis with concentrated acids (Denis, 1910). This is due to secondary decomposition and much of the additional amount of ammonia probably originates from the decomposition of tryptophane and cystine. The tables of data for the rate of hydrolysis of amide nitrogen by 1.0 N and more concentrated hydrochloric acid show this slow secondary decomposition very clearly.

0.2 N sulfuric acid is less effective as a hydrolyzing agent than 0.2 N hydrochloric acid, the curve lying between those showing the rate of hydrolysis by 0.1 and 0.2 N hydrochloric acid for most of its length (Chart I).

With alkaline hydrolyzing reagents secondary decomposition, especially that of arginine, contributes a much larger proportion of the total ammonia than is the case with acid reagents. The rate of hydrolysis of the amide nitrogen appears to be even more

rapid with 0.2 N sodium hydroxide than with 0.2 N hydrochloric acid. The rate at which the ammonia is set free shows that three distinct phases of the reaction exist. The greater part of the ammonia is liberated rapidly and this phase represents the hydrolysis of the amide nitrogen. When all the amide nitrogen is set free, ammonia comes off at a much slower but quite steady rate for a number of hours. This phase probably represents the decomposition of arginine. Finally when an amount of nitrogen has been liberated corresponding roughly with the known amount of amide nitrogen and one-half the arginine nitrogen, the rate of liberation of ammonia becomes exceedingly slow. This final phase probably represents the decomposition of amino-acids other than arginine. All of these reactions probably proceed simultaneously but become evident on the curve as they are successively terminated.

0.2 N barium hydroxide is a more rapid hydrolyzing agent than 0.2 N sodium hydroxide with respect to the amide nitrogen but causes secondary decomposition at a slower rate (Chart II).

By hydrolyzing gliadin with dilute acid it is possible to remove nearly all of the amide nitrogen and at the same time split very few of the peptide bonds. It is therefore possible to secure products from gliadin which still retain a large proportion of the peptide bindings unbroken and which are practically free from amide nitrogen. The study of these products will form the subject of a later paper.

While our experiments with 1.0 and 2.0 N acid were not prolonged until hydrolysis had been entirely completed, we have no reason to doubt that it would be possible to push the hydrolysis, at any rate, very nearly to completion. Whether or not weaker acid reagents can eventually split all the peptide bonds at boiling temperature is not certain.

Hydrolysis of gliadin as measured by the appearance of amino nitrogen is practically complete when the protein has been boiled about 20 hours with 20 per cent hydrochloric acid or for about 50 hours with 4 N hydrochloric acid.

The curves which show the rate of hydrolysis of gliadin by the stronger acid-hydrolyzing reagents have no irregularities, but indicate that if hydrolysis is sufficiently prolonged it will eventually become complete. The peptide bonds of the protein are

broken successively in a perfectly smooth manner, the process continuing until the entire molecule is hydrolyzed to amino-acids. The action of acid-hydrolyzing agents is thus in sharp contrast to that of enzymes; pepsin, for example, as has been shown by the work of Frankel (1916), hydrolyzes a protein to a certain point at which action ceases. It thus is evident that there are portions of the protein molecule which are resistant to the action of this enzyme. Precisely the same remarks apply to the action of trypsin, but it is clear that they cannot be applied to the hydrolytic action of strong acids on the protein. A fundamental difference, therefore, exists between the hydrolysis of a protein by acids on the one hand, and by enzymes on the other hand (Chart III).

The initial rate at which the peptide bonds of gliadin are split by alkaline reagents is much more rapid than the initial rate at which they are split by acids of equivalent concentration. Apparently there are certain peptide bonds in the protein molecule which are extremely susceptible to attack by alkali. These break up very rapidly and the process then continues at a rate resembling that observed when equivalent concentrations of acid are used.

The rate of hydrolysis effected by barium hydroxide is much more rapid than that by an equivalent concentration of sodium hydroxide. No explanation of this observation can be suggested but it appears that the stability of the peptide bond is in some way influenced by the presence of the divalent metallic ion (Chart IV).

Gliadin was prepared according to the directions of Osborne and Harris (1906) with the exception that no sodium chloride was added to the water used for precipitating the concentrated alcoholic solution. In the air-dry condition this preparation analyzed as follows:

	per cent
Nitrogen	16.03
Moisture	8.64
Ash	0.18
Nitrogen, ash- and mosture-free	17.58

The hydrolyzing reagents were made up of such a concentration that when 20 cc. of gliadin solution (1 gm.) were added to 80 cc. of reagent the concentration would be exactly 0.1 N, 0.2 N, etc.

The procedure was as follows: 80 cc. portions of hydrolyzing reagent were pipetted into 200 cc. Pyrex flasks and warmed under a reflux condenser on an electric hot-plate to about 90°C. 20 cc. of 5 per cent gliadin solution in 70 per cent alcohol, of which the concentration was controlled by nitrogen determinations, were then run in. By this means a solution of 1 gm. of gliadin in 100 cc. of 0.1 N, 0.2 N, etc., hydrolyzing reagent was conveniently obtained and the hydrolysis begun, from a definite point of time.

At the end of the desired period of boiling the contents of the flasks were rapidly cooled and treated with sodium hydroxide until a precipitate which separated during the addition of the alkali had just redissolved. At this point the solutions were slightly acid to phenolphthalein. Magnesium oxide was then added in excess and the ammonia distilled into tenth normal acid. The distillation was contined until about 60 to 70 cc. of residue remained. This was made up to 100 cc. and aliquots of 10 cc. were withdrawn, after careful shaking, for amino nitrogen determinations by the Van Slyke method. Total nitrogen was also determined in a 25 cc. aliquot to serve as a check against the ammonia determination.

When alkaline hydrolyzing agents were used the procedure was somewhat modified. An adapter on the upper end of the reflux condenser was dipped into a flask containing dilute sulfuric acid to catch the ammonia. The hydrolysis was carried out in 750 cc. flasks and at the end of the hydrolysis period sufficient acid was run in through a dropping funnel to neutralize the contents. The condenser was then adjusted to the distilling position without disconnecting at any point and water and excess of magnesia were added to the flask. Distillation of the ammonia could thus be effected without loss. It was found impossible to titrate the ammonia obtained by alkaline hydrolysis of gliadin on account of foul smelling decomposition products in the distillate which rendered the end-point uncertain. Moreover, hydrogen sulfide was found in the distillate. The nitrogen in the distillate was therefore determined by the Kjeldahl method.

All determinations were made in duplicate.

During the hydrolysis of gliadin by acids of different concentrations certain precipitation and color reactions occurred which require mention.

With 0.1 N hydrochloric acid there was no distinct separation at any period of the hydrolysis although the solution became opalescent. With 0.2 N and higher concentrations of acid a precipitate began to appear soon after boiling had begun, the more rapidly, the higher the concentration of the acid up to 4.0 N. This precipitate slowly dissolved as hydrolysis proceeded, 16 or more hours being necessary with 0.5 N acid but only about 3 hours with 4.0 N hydrochloric acid.

There was no marked color change save a slight darkening of the yellow solution, on long hydrolysis with any acid less concentrated than 2.0 N.

With 2.0 N hydrochloric acid after 65 hours boiling the solution had become pale green, while with 4.0 N acid a green color appeared in the clear solution after 5 hours boiling and in 16 hours the solution was emerald green.

With 20 per cent hydrochloric acid there was no precipitation but the pink Liebermann reaction developed a few minutes after adding the protein to the hot acid. This color soon changed to yellow-brown and in 4 hours the solution was distinctly green. Longer periods of hydrolysis changed the color to brown but no separation of black humus occurred.

The changes observed when 0.2 N sulfuric acid was used resemble those noted with 0.1 N hydrochloric acid and those with 4.0 N sulfuric acid were very similar to the changes observed with 2.0 N hydrochloric acid.

No precipitation of protein material or color changes were observed during hydrolysis with alkalies, the color remaining yellow throughout. Considerable inorganic material derived from the flasks was observed especially when alkaline hydrolysis was prolonged.

The temperature at which the hydrolyses occurred was influenced by the presence of the alcohol and ranged from 93 to 96° with the weaker hydrochloric acid reagents. Loss of alcohol, probably through the formation of ethyl chloride, occurred with hydrochloric acid stronger than 2.0 N and the temperature consequently rose on prolonged hydrolysis.

The non-appearance of humus in the solutions which had been hydrolyzed by 20 per cent hydrochloric acid is due to the small concentration of protein in the solution. When 2 gm. or more of gliadin were boiled in 100 cc. of 20 per cent hydrochloric acid, sufficient humus was formed to appear as a precipitate.

Tables I to XIII give the percentage of the total nitrogen of gliadin found as ammonia nitrogen and the percentage of the total available amino nitrogen found as amino nitrogen after hydrolysis as noted. The total available amino nitrogen of gliadin was taken as 57.3 per cent of the total nitrogen (Van Slyke, 1912). This figure was confirmed by an estimation of the total amino nitrogen obtained from a sample of the gliadin used in this work.

TABLE I

0.027 N HCl at 93–94°C.

Time.	Total N as ammonia N.
hrs.	per cent
5	7.7
15.5	13.2
20	14.3
28	15.5
44	16 8
70	17.7
118	19.5

TABLE II

0.1 N HCl at 93–94°C.

Time.	Total N as ammonia N.	Total amino N.
hrs.	per cent	per cent
1	8.5	
2	14.4	3.7
3	17 4	
4	18.8	3 5
8	21.7	5.3
11	22.9	6.3
17	23.4	7.6
26	24.1	13 6
40	24 2	17.3

TABLE III.

0.2 N HCl at 93–94°C.

Time.	Total N as ammonia N.	Total amino N.
hrs.	per cent	per cent
0.5	10.7	
1	14.0	2.5
3 5	21.8	4.6
6	23.8	
16	23.6	15.6
24	23.9	23.3
40	24.4	30.0

TABLE IV.

0.5 N HCl at 93–94°C.

Time.	Total N as ammonia N.	Total amino N.
hrs.	per cent	per cent
0 5	17.4	4.3
1	21.0	6.5
2	23.4	12.3
4	24.5	29.7
16	24 6	37.9
22	24 9	40.2
24	24.9	56.8
40	25 0	

TABLE V.

1.0 N HCl at 94–95°C.

Time.	Total N as ammonia N.	Total amino N.
hrs.	per cent	per cent
0 5	22.2	9.6
1	23.6	11.5
2	24.2	31.2
7	25.0	42.8
12	24.8	46.7
14	24.9	49.2
16	25.0	51.1
18	24.9	57.5
22	25.0	59.9
27	25.0	69.1
40	25.1	76.9
49	25.3	83.7
69		

TABLE VI.

2 0 N HCl at 94–96°C.

Time.	Total N as ammonia N.	Total amino N.
hrs.	per cent	per cent
1	24.7	21.7
3.3	24.9	39.5
5	25.1	43.7
7	24.9	50.2
9	25 0	55.5
12	25.2	62.1
17	25.5	74.7
24	25.4	79.5
45	25.2	84.0
65	25.5	88.5

TABLE VII.

4 0 N HCl at 98–104°C.

Time.	Total N as ammonia N.	Total amino N.
hrs.	per cent	per cent
0.5	24.9	30.4
1	24.8	45.0
2.5	25.3	56.6
4.5		70.8
5	25.4	75.0
8		83.5
13	25.4	89.3
16		90.0
24	25 5	95.3
40		96.9
48	25.5	98.2
72	25.5	98.5

TABLE VIII.

20 per cent HCl at 102–110°C.

Time.	Total N as ammonia N.	Total amino N.
hrs.	per cent	per cent
1	25.2	59.2
2		71.5
3	25.2	80.1
4	25.3	82.1
5	25.4	88.2
7.5	25.5	91.8
11	25.5	95.9
15	25.4	97.6
19		98.7
40	25.6	98.8

TABLE IX.

0.2 N H_2SO_4 at 93–94°C.

Time.	Total N as ammonia N.	Total amino N.
hrs.	per cent	per cent
1	9.4	
2	14.8	
5	21.6	
6	22.6	
12	23.3	4.9
16	24.4	
24	24.7	
48	25.2	22.1
71	25.6	29.8

TABLE X.

4.0 N H_2SO_4 at 96-98°C.

Time.	Total N as ammonia N.	Total amino N.
hrs.	per cent	per cent
1	25.0	21.5
3	25.1	40.6
5	25.2	46.9
7		56.2
11	25.3	64.9
17	25.6	76.9
24	25.5	82.0
41	25.5	86.3
65	25.6	94.0

TABLE XI.

0.2 N NaOH at 93-94°C.

Time.	Total N as ammonia N.	Total amino N.
hrs.	per cent	per cent
1	17.8	8.4
2	22.7	10.1
3	24.7	
4	25.5	13.4
5	26.4	14.6
6	27.2	17.2
8	29.0	18.1
12	29.2	
17		20.5
46	29.9	26.2
72		31.8

TABLE XII.

1.0 N NaOH at 94-95°C.

Time.	Total N as ammonia N.	Total amino N.
hrs.	per cent	per cent
1	26.8	17.1
2	28.1	24.0
3	28.5	28.9
5	29.2	33.4
6		34.6
8	30.0	39.0
15		47.3
24	29.8	60.3

TABLE XIII.

0.2 N Ba $(OH)_2$ at 93-94°C.

Time.	Total N as ammonia N.	Total amino N.
hrs.	per cent	per cent
1	23.7	15.0
3	26.3	21.8
5	26.7	26.7
7		29.3
8	27.1	
17	29.0	39.3
46	30.1	56.9

CHART I. Amide hydrolysis of gliadin by acids.

CHART II. Amide hydrolysis of gliadin by alkalies.

CHART III. Peptide hydrolysis of gliadin by acids.

CHART IV. Peptide hydrolysis of gliadin by alkalies.

SUMMARY.

1. The rate at which gliadin is hydrolyzed at boiling temperature by the following reagents has been investigated: 0.027, 0.1, 0.2, 0.5, 1.0, 2.0, 4.0 N hydrochloric acid; 20 per cent hydrochloric acid; 0.2, 4.0 N sulfuric acid; 0.2, 1.0 N sodium hydroxide; 0.2 N barium hydroxide.

2. The liberation of ammonia from gliadin, presumably amide hydrolysis, is readily effected at boiling temperature by very dilute acid or alkali. The ammonia is set free with great rapidity by the stronger acid reagents.

3. Peptide hydrolysis is nearly completed by boiling gliadin with 20 per cent hydrochloric acid for 20 hours or with 4 N hydrochloric acid for 50 hours. Much longer periods are required when less concentrated acid reagents are used.

4. Alkalies hydrolyze gliadin more rapidly in the early stages of hydrolysis than equivalent concentrations of acids in respect to both amide and peptide bindings. Due to secondary decom-

position, the amount of ammonia set free by alkalies is considerably greater than that liberated by acid reagents.

5. Barium hydroxide hydrolyzes gliadin more rapidly than sodium hydroxide of equivalent concentration in respect to both amide and peptide bindings.

6. By the use of varying concentrations of acid-hydrolyzing reagents a picture of the hydrolysis of gliadin has been obtained from the splitting of the first bonds to the completion of the reaction. Acid hydrolysis is thus shown to be a continuous process proceeding from first to last without marked interruption due to the existence of stable complexes, and is therefore clearly distinguished from enzymatic hydrolysis.

BIBLIOGRAPHY.

Andersen, A. C., *Biochem. Z.*, 1915, lxx, 344.
Andersen, A. C., and Roed-Müller, R., *Biochem. Z.*, 1915, lxx, 442.
Braconnot, H., *Ann. chim. phys.*, 1820, xiii, 113.
Dakin, H. D., *Biochem. J.*, 1918, xii, 290.
Dakin, H. D., *Biochem. J.*, 1919, xiii, 398.
Denis, W., *J. Biol. Chem.*, 1910, viii, 427.
Dunn, M. S., and Lewis, H. B., *J. Biol. Chem.*, 1921, xlix, 343.
Fischer, E., and Abderhalden, E., *Ber. chem. Ges.*, 1904, xxxvii, 3071.
Frankel, E. M., *J. Biol. Chem.*, 1916, xxvi, 31.
Harding, V. J., and Fort, C. A., *J. Biol. Chem.*, 1918, xxxv, 29.
Henriques, V., and Gjaldbäk, J. K., *Z. physiol. Chem.*, 1910, lxvii, 8.
Henriques, V., and Gjaldbäk, J. K., *Z. physiol. Chem.*, 1911, lxxv, 363.
Hofmeister, F., *Ergebn. Physiol.*, 1902, i, *1 te Abt.*, 759.
Jones, D. B., and Johns, C. O., *J. Biol. Chem.*, 1921, xlviii, 347.
Johnson, T. B., and Burnham, G., *J. Biol. Chem.*, 1911, ix, 331.
Mathieu, H., *Compt. rend. Acad.*, 1909, cxlviii, 1218.
Mulder, G. J., *J. prakt. Chem.*, 1839, xvi, 290.
Nasse, O., *Arch. ges. Physiol.*, 1872, vi, 589.
Northrop, J. H., *J. Gen. Physiol.*, 1918–19, i, 607.
Osborne, T. B., and Harris, I. F., *Am. J. Physiol.*, 1906–07, xvii, 223.
Osborne, T. B., Leavenworth, C. S., and Brautlecht, C. A., *Am. J. Physiol.*, 1908–09, xxiii, 180.
Osborne, T. B., and Nolan, O. L., *J. Biol. Chem.*, 1920, xliii, 311.
Pittom, W. W. P., *Biochem. J.*, 1914, viii, 157.
Siegfried, M., *Z. physiol. Chem.*, 1905, xliv, 85.
Sörensen, S. P. L., *Biochem. Z.*, 1908, vii, 45.
Thierfelder, H., and von Cramm, E., *Z. physiol. Chem.*, 1919, cv, 58.
Van Slyke, D. D., *J. Biol. Chem.*, 1911, ix, 185.
Van Slyke, D. D., *J. Biol. Chem.*, 1912, xii, 295.
Walters, E. H., *J. Biol. Chem.*, 1912, a, xi, 267.
Walters, E. H., *J. Biol. Chem.*, 1912, b, xii, 43.

ON ABNORMAL MILK AND ON THE INFLUENCE OF AN ASEPTIC UDDER INFLAMMATION ON THE COMPOSITION OF THE MILK.

By B. SJOLLEMA and J. E. VAN DER ZANDE.

(From the Chemical Laboratory of the Veterinary University of Utrecht, Utrecht, Holland.)

In 1921 we analyzed a number of abnormal milks which possessed the chemical composition that is considered to be characteristic for milk from udders with streptococcic infections.

In several of these samples streptococci were absent. The number of leucocytes in nearly all, with or without streptococci, was very high.

These results gave us the impression that the rôle usually ascribed to these bacteria in producing udder inflammations and the secretion of abnormal milk, is exaggerated. We examined this point by producing a sterile inflammation of one of the quarters of a normal udder and by comparing the milk of the thus treated quarter with that of the normal quarters as well as with the milk of the quarter before the inflammation. For this purpose a solution of 0.2 per cent silver nitrate was under sterile conditions injected into one quarter (R. F.) of a healthy milch cow in full lactation.

We also used a second way to find out whether the streptococci are as important in causing abnormal milk as is usually believed. This second method consisted in the analyzing of milks of udder-diseased cows brought to the abattoir and by making a bacteriological and anatomical examination of the udder tissue.[1]

The interesting paper of Baker and Breed (1) supported our opinion that the importance of the streptococci is not so great as is generally believed. Notwithstanding this fact, they concluded

---

[1] We are indebted to Mr. H. S. Frenkel for the examination of the udder tissues.

that the abnormalities of their samples of milk were due to strepto-
cocci. We believe that another interpretation is not excluded.
From eleven samples with very high pH values (6.92 to 7.0) they
found one free from streptococci. Again in four out of ten
samples with high pH values (6.84 to 6.92) these bacteria were
absent. The same result obtained with five samples out of
thirteen with pH values between 6.76 and 6.84.

We cannot see the impossibility of the abnormality of these
samples being caused by any other agent than streptococci.

Methods.

We generally used the methods of the Dutch Codex Alimen-
tarius for milk (3rd edition). The titratable acidity was deter-
mined with 0.1 or 0.25 N NaOH, using phenolphthalein as indi-
cator and expressing the acidity in the number of cc. of 0.25 N
NaOH used for 100 cc. of milk.

The oxalate acidity was estimated in the same way after
adding 4 cc. of a neutral 10 per cent solution of potassium oxalate
per 25 cc. of milk.

We think it worth while to point out the fact that milk of
abnormal acidity is detected easily by adding a phenol red solution
to the oxalated milk, as normal milk gives the transition tint of
phenol red.

The chlorides were estimated by adding nitric acid to the milk,
filtering, and using the Volhard method in the filtrate as
described in the above mentioned Codex.

For lactose we usually chose the method of Folin and Denis.[2]
For very abnormal samples this method cannot be used, as a
persistent blue-violet color appears during the boiling.

We then applied the iodometric titration (of the excess of
$CuSO_4$) after boiling with Fehling's solution. For the estimation
of the total protein we used the Kjeldahl method. The casein
was precipitated with a saturated solution of potassium-aluminium
sulfate. With abnormal milks neither this method nor the other
methods recommended for the elimination of casein, give exact
results. The calcium was determined in the protein-free fil-
trate (trichloroacetic acid was used to remove the proteins) by pre-

[2] Folin, O., and Denis, W., *J. Biol. Chem.*, 1918, xxxiii, 521.

cipitating the faintly acid solution (indicator methyl orange) with
ammonium oxalate, contrifuging, washing, and titrating with
$KMnO_4$ solution.

For the carbonic acid estimation we used the apparatus of
D. D. Van Slyke, acidifying with lactic acid.   As recommended
by Van Slyke and Baker[3] the CO_2 was absorbed with a solution
of NaOH.   The tryptophane was determined colorimetrically by
the method given by Fürth and Nobel (2) by means of the reac-
tion of Voisenet.   The number of leucocytes was estimated by
the method of Baker and Breed (3).

For the sediment estimation we used the well known Tromms-
dorff tubes for 10 cc. of milk with capillair.   In 1921 we used
for the determination of the non-protein nitrogen metaphosphoric
acid; in 1922 we precipitated the proteins with sodium tungstate
and sulfuric acid (Folin and Wu).   The filtrate was nesslerized
after destruction.   The pH values were found with the indicator
method as described by Baker and Van Slyke (4).   Instead of
brom-cresol purple we used phenol red as we preferred the latter.
We must remark that the indicator method does not give exact
figures with very abnormal milks nor with colostrum.   These
milks are usually colored.

DATA AND DISCUSSION.

Table I gives the data of the milk samples from the milch cow
which was treated with silver nitrate solution.   The right fore
quarter was injected on March 8.   The samples Nos. 381 R. F.
and L. F. were taken March 9, about 14 and 24 hours after
injection.   The milk from the right fore quarter was abnormal
both times, as is seen from the data of the table.

Again the following day the composition and appearance of
the milk from the injected quarter were abnormal, whereas the
other quarters gave normal milk.   Gradually the abnormality of
the milk diminished; for instance, the composition of the milk of
March 16, though still abnormal, differed less from normal milk
than that of the previous days.   The appearance of this milk
was almost normal.   The quantity of milk drawn from this
quarter, which was very small in the first days after the injection,

[5] Van Slyke, L. L., and Baker, J. C., J. Biol. Chem., 1919, xl, 335.

TABLE I.

Milk from a Cow Which Was Treated with Silver Nitrate Solution and with Turpentine.

The R.F. quarter injected with silver nitrate on Mar. 8.

Nos. 381a, 382a, 383a, 385b, 386a, 387a, and 388a were drawn from the quarter injected with silver nitrate.

Nos. 337, 343, and 375 were taken before the injection.

Nos. 381b, 382b, 383b, 382a, 385a, 386b, 387b, and 388b were taken from other quarters than that injected with silver nitrate.

Nos. 349, 351, 358, and 363 were taken some days after the injection with turpentine.

Date	No.	Quarter	Acidity	Oxalate acidity	pH	Sediment (per cent)	Leucocytes	Non-protein nitrogen (mg per 100 cc)	Chlorides (mg per 100 cc)	Lactose (per cent)	Total proteins (per cent)	Casein (per cent)	CaO (mg per 100 cc)	CO_2 in 2 cc of milk	Tryptophane in serum (mg per 100 cc)
Mar. 9	381a	R.F.	2.5			0.35	Somewhat too many.	53.3				2.7	120.0		109.6
" 9	382a	R.F.	3.3			2.2			298.4						348.0
" 10	383a	R.F.	3.7			5.0			280.0						300.0
" 13	385b	R.F.	5.3	1.3	6.98–7.06	Very much.	Too many to count.				8.05	3.8	152.0	0.477	
" 14	386a	R.F.	5.35			4.0					5.75	3.1	199.7	0.55	171.0
" 16	387a	R.F.	4.8	0.0	6.75	0.5			153.6	2.7	3.53	2.6	196.4	0.3	90.0
" 17	388a	R.F.	4.8		6.82										
Jan. 3	337*	4 quarters.	7.0			0.25	Normal.	28.8	104.0	4.43	2.39		245.0		Normal.
" 11	343*	4	7.6	2.2		0.3	"	32.9	90.9	3.8	2.92				"
Feb. 24	375	R.F.	8.6	4.25		0.35	"		88.4						
Mar. 9	381b	L.F.	7.0			0.1	"								

Date	No.									
Mar. 9	382b	R.H., L.F., L.H.		6 5 6 6	0 6	Many.	84.3			
" 10	383b	R.H.								
" 13	385a	L.F., L.H.	8 6 4 2			Normal.				
" 13	385c	R.H.	8 86 4 6		0.25	Somewhat too many.				
" 14	386b	R.H.	8 24			Normal.				
" 16	387b	R.H.	8 0		0.2		106.6			
" 17	388b	R.H.	5 8			Normal.	96 6			
Jan. 14	349†	4 quarters.	8 4 3 2		0 25	"	79.8	235.0		22.0
" 16	351†	4	10 3 4 5		0 3	"	72.2	205.7 0.13		21.0
" 24	358	4	8 2		0 4	"				
" 31	363	4			0 25					

* The milk of the 4 quarters was analyzed separately. The differences were of no importance.

† The sediment was determined separately in the 4 quarters with the same result.

had largely increased. However, on March 19 and later, it was impossible to get any milk from this quarter notwithstanding the fact that it had been milked regularly at the same hours as the other quarters. We could never find streptococci in the milk of the treated quarter.

The figures of Table I show that the milk of this quarter resembles milk of quarters with streptococcic infection. This will be confirmed by some of the data given in Table II. It applies to different constituents: chlorides, lactose, total proteins, carbonic acid, calcium, and tryptophane, and holds good also for the acidity, pH values, number of leucocytes, and sediment. The non-protein nitrogen, estimated twice, was once too high. The milk of the other quarters had normal appearance, acidity, and pH value; also the content of the chlorides, number of leucocytes, and amount of sediment were normal; with the exception, that the sediment of the R. H. quarter was on March 10 a little too high.

The low acidity of the milk of the R. F. quarter was to be expected, as the composition of milk from inflamed udders approaches that of blood plasma (5). Nevertheless, we have found in several milks from quarters with streptococcic infections high as well as low acidities. The high acidity in these samples may be caused partly by the acid formation of these bacteria and partly by a high percentage of globulins, as in colostrum.

Later on we will discuss the tryptophane figures. Our experiment proves that other affections than streptococcic invasions can produce the same changes in milk. Therefore, aseptic and bacteriological inflammations have in general the same influence on the chemical composition of milk. Our results make it more probable that streptococcic infections of the udder are usually originated by non-bacteriological lesions; namely, that the streptococcic invasion is secondary.

Table I also gives the figures of some samples of milk of the same cow taken before the treatment with silver nitrate. There is no reason to discuss these figures as they are normal. The same cow was used 2 months earlier for another experiment; i.e., to examine whether an aseptic abscess in other parts of the body than the udder, in this case in the region of the neck, influences the composition of the milk. The abscess was produced by sterile injection of turpentine. It developed slowly to a

rather large size. Neither during the development nor after-
wards was the composition of the milk much affected. Only
once did the milk of one of the quarters give a sediment that was
a trifle too high (it was 0.6 per cent). Sometimes the acidity
was too high; *i.e.*, 10.3 (it was determined within half an hour
after the drawing). Instead of 90 to 100 mg. we found in some
of the samples 80 and 72 mg. of Cl. The milk with 72 mg. of Cl
contained 0.14 cc. of CO_2 in 2 cc. Once (4 days after the injection
with turpentine), we found in the milk a great many corpuscles
(cells) with round nuclei of different sizes.

Concerning the results formerly (in 1912) found in this labo-
ratory in milk of cows suffering from different diseases, we men-
tion merely that disturbances of the digestion and external
diseases frequently influenced the composition of the milk (6).
Chlorides and total proteins were increased, lactose and acidity
decreased.

The alcohol test, *i.e.* mixing the milk with an equal volume of
70 per cent alcohol, often gave a positive result; namely, the form-
ing of small clots, much smaller than those which are formed
when this test is used for sour milk. Later on we got similar
results with milk from cows suffering from lung tuberculosis from
an abscess on the jaw and from inflammation of the kidneys. In
a case of pyelonephritis the acidity was too high. The number
of leucocytes was usually normal, sometimes a little greater.

In contrast to the milk of udder-diseased cows there was no
great difference between the milk of the four quarters. Our
experiment with turpentine injection, in which the temperature
of the animal rose only slightly, proves that an aseptic inflam-
mation in any other part of the body had only very little influence
on the milk. It may be that toxic substances formed by bacteria
can cause the secretion of abnormal milk.

Table II gives some of the results, which gave us in 1921 the
impression that the importance of streptococcus is not so great as is
usually believed. Several of the samples were highly abnormal
both in appearance and composition. We usually analyzed the
milk of each quarter separately. In several cases the milk of the
other quarters was examined at the same time; not all these
results are mentioned in the table. Table II gives the data of
the milks without streptococci. No. 112 contained only a small
number, Nos. 164 and 142 were rich in streptococci.

TABLE II—*Abnormal Milks, All without Str...*

No.	Acid-ity.	Oxa-late acid-ity.	pH	Sediment.	Leucocytes.	Non-protein nitro-gen.	Ch id
						mg. per 100 cc.	*mg 10(*
92	2.6			1.4 per cent.		34.0	2:
94	5.2					40.5	1(
96	5.6	1.3		1 3 per cent.		34.5	
	6.4	1.8		2.1 " "		34.5	
97	3.36	−0.8		± 3 " "		52.3	2(
98	4.8	−0.2	6.82–6 9	0.8 " "		55.4	1'
110	6.0	1.2	6.75	0 2 " "	A great number.	46.6	1(
112	6.02	1.6	6.67–6.75	0.9 " "	" " " A few streptococci.	37.0	1
131	6.2	3.7		3.5 " " A little blood.		68.6	1
154	6.4	±0		±2.5 cc.	Full of leucocytes.	60.6	
161	6.8	2.6		0.6 per cent.		38.1	1
162	7.8	4.8		± 3 cc.	Full of leucocytes.	60.2	:
167	±4.0	±0.6		± 3 "	A great number.	46.7	:
170 and 171	3.2	0.2	6.98	2.3 per cent.	" " "	40.0	
180	2.8	0.0	± 6.9–6.98	A little blood. ± 1.7 per cent.	" " "	39.2	
206	5.4	2.2		±5 per cent.	Full of leucocytes.	133.3	:
207	4.0	0.8		±1.5 cc. A little blood.		63.4	
208	5.0	−1.4		±4 per cent. A little blood.	Full of leucocytes.	78.4	
230	5.1	0 85	6.75–6.82	0.4 per cent.	Large number.		
230a	5.1	0.85		0.4 " "		27.6	

:ept Nos. 112, 142, and 164.

Fat.	Casein.	CaO	Trypto-phane in serum.	Quarter.	Remarks.
per cent	*per cent*	*mg. per 100 cc.*	*mg. per 100 cc.*		
		206.4			Milk from 4 quarters (Cow 456); appeared nearly normal; a few small clots.
				L.F.	From Cow 456. The milk of the L.H.
				L.F.	and R.H. quarters contained strep-
				R.F.	tococci.
		88.0		L.F.	
2.6		252.0	High.		14 days after calving.
2.25	2.47	214.0		L.H.	14 " " " Injected with
2.55	1.18	198.7	Normal.		streptococcic serum before the examination of milk.
2.7	1.085		High.		13 days after calving. Milk from 4 quarters. 12 days later streptococci present.
		55.6		R.H.	Appeared very abnormal. Other quarters (*i.e.* L.F.) gave normal milk.
4.0		166.3		R.H.	14 days after calving. Appeared very abnormal.
2.1	3.1	99.3	Very high.	L.H.	5 days after calving. Appeared very abnormal.
1.048		130.8			4 quarters, at the end of lactation appeared very abnormal.
			High.	R.H. and R.F.	From Cow 456, some days earlier streptococci were present.
			"		4 quarters, 14 days after calving, appeared abnormal.
4.8		189.7		R.F.	In full lactation, appeared very abnormal, lesion of teat; one of the quarters (L.H.) gave milk with some streptococci.
				L.F.	Same cow as No. 206.
6.2		263.8		R.H.	" " " " 206. (catalase 5.2).
		137.7	Normal.	R.F.	
		137.7	14.5	R.F.	In full lactation, no diseases to be found.

TABL[...]

No.	Acidity.	Oxalate acidity.	pH	Sediment.	Leucocytes.	Non-protein nitrogen.	C[...]
						mg. per 100 cc.	*m[...]*
230b	6.24	1.73		0.4 per cent.		30.0	1[...]
230c	6.2	1.43	6 65-6.72	0 5 " "			
230d	5.73	1.33	6 6 -6.65	0.4 " "		34.3	1[...]
255	2.4				± 2,480,000 leucocytes per cc.		
256	3.6		±6 98		±1,000,000 leucocytes per cc.		
257	5.4		6 82-6.9		±280,000 leucocytes per cc.		
261	2.0	−2.05					
260	2.0	−1.95					
263	2.0	−2 0		± 3.0 cc.	±20,000,000		[...]
262	3.8	−1 8		±1 5 "	± 2,320,000		[...]
265	1.2	−1 68	6.9 -6.98		± 3,240,000 leuco-	45.4	[...]
264	2.16	−0 88			± 1,200,000 cytes.	41.0	1[...]
267	4 61	±0 1	6.75-6.82		± 450,000		1[...]
266	5.93	±0 27	6.67-6.75		± 160,000		[...]
297	±6.4					73.7	
					Milk samples		
164	4 8	1 2	6.8 -6.9	± 1.5 cc.			[...]
142	5.6	0 8	6.75-6.82	0.5 per cent			[...]

ed.

Fat.	Casein.	CaO	Trypto-phane in serum.	Quarter.	Remarks.
per cent	*per cent*	*mg. per 100 cc.*	*mg. per 100 cc.*		
		179.0	16.6	L.F.	Same cow as No. 230a; the milk yields of this cow were abnormally small.
				R.H.	Same cow as No. 230a.
		186.0	16.3	L.H.	" " " " 230a.
				R.H.	Cow with inflammation of the throat and of the udder; however, no clinical mastitis could be detected by the veterinarians.
				L.H.	Same cow as No. 255; appeared very abnormal, like No. 255.
				L.F.	
				R.H.	3 days later than No. 255, appeared very abnormal. The clots make the determination of the leucocytes unreliable.
				L.F.	Same cow as No. 255.
Very little.		119.0		R.H.	2 days later than No. 261, appeared very abnormal.
		210.8		L.F.	Same cow as No. 255, No. 262 without reductase.
		173.5		R.H.	2 days later than No. 263; same cow as No. 255.
				L.F.	Appeared less abnormal, catalase of both = 7.
		274.4		R.H.	2 days later than No. 265; same cow as No. 255.
		203.0		L.F.	Reductase test discolored within 2 hours.
		298.0			
streptococci.		101.0	3×normal	R.H.	10 days after calving.
		188.0		L.H.	

The table mentions whether or not the milk was produced soon after calving. From the remarks made in the last column it may be seen that not infrequently at the same time that abnormal milk without streptococci was drawn from one quarter, other quarters gave milk with some streptococci. We also found that a quarter giving milk without streptococci 1 day produced at another time milk containing these bacteria in great numbers.[4] One of the striking features was that *in milk with a low acidity (less than about 3) streptococci were never present.* Perhaps one might conclude therefrom, that when the milk is highly abnormal it is no adequate medium for these bacteria. Abnormal milks, with or without streptococci, show acidities low (4 to 6) or normal (7 to 8). This was found in milks analyzed when quite fresh (*i.e.* within half an hour). The acidity is therefore no good criterion for streptococcic invasions in the udder. The high acidity sometimes found in streptococci-containing milk may in some cases be explained by the character of colostrum, the milk still possessed as colostrum has a high acidity; *i.e.*, of 17. In other cases it may be caused by the acids formed by the streptococci.

We found in sterilized milk, by infecting with *Streptococcus mastitidis bovis* and keeping at 37°C. for 3 days, the acidity increased from 8 to 24.4 and a second time from 8.4 to 29 in 2 days. Infection of sterilized milk with *pyogenes* had no or only a negligible influence on the acidity. We wish to point to the result of the generally used reduction test with methylene blue obtained on Sample 262. There was no reduction; *i.e.*, no discoloration, even in 24 hours.

In our abnormal samples without streptococci, as well as in those containing these bacteria, we found the *chlorides and tryptophane greatly increased, the lactose decreased.* As udder diseases often occur shortly after calving, it is necessary to take into account that the chemical criteria for abnormal milk give values for colostrum that resemble more or less those for milk of diseased udders.

[4] A more detailed report of our work is published in *Tydschr. Vergelijkende Geneesk. (J. Comparative Med.)*, 1922, vii, official organ of the Society for the Knowledge of Milk.

The *non-protein nitrogen* gave with normal milks the same values when the proteins were precipitated with tungstic acid (Folin) or when metaphosphoric acid (25 per cent) was used for this purpose. This does not hold good for colostrum nor for abnormal milks. We will try to find the reason for this difference.[5] The non-protein nitrogen of abnormal milks is frequently higher than of normal milk, *i.e.* about 60 mg. instead of about 30 to 40 mg., as well as when streptococci are present in the absence of these bacteria. Nevertheless, the determination of this value is no sharp method for the detection of abnormal milk. In the above mentioned sample of milk, which was infected with streptococci we found no increase of the non-protein nitrogen. On the contrary, infecting sterilized milk with *pyogenes* raised the figure from 30 to 200 mg.; ammonia alone was almost formed. From the cow injected with silver nitrate solution only once did we determine the non-protein nitrogen; it was 53.3 mg. in 100 cc. The amount of non-protein nitrogen which we found in several samples of colostrum was always higher than in normal milk, usually 60 to 120 mg. in 100 cc.

In venous blood from the jugular vein of cows we found figures between 23 and 36 mg., shortly after parturition. That these figures are much lower than those of colostrum may be connected with the much higher content of nitrogenous substances in colostrum than in blood.

Concerning our *tryptophane* results obtained in 1921 and with the milk of the cow which was injected with silver nitrate, the following may be mentioned. Normal serum of milk, after precipitating the casein and the fat with a saturated solution of potassium-aluminium sulfate, contains about 14 to 20 mg. of tryptophane in 100 cc. In abnormal milks the amount is usually ten to fifteen times as high. *Of colostrum* prepared in the same way the tryptophane content is usually more than 0.5 gm. in 100 cc. The amounts of tryptophane, we found in abnormal milks, according to our results, approach that for blood serum. Milk contains no free tryptophane either in the ultrafiltrate or in the filtrate obtained after precipitating the proteins. Tryp-

[5] Some years ago in this laboratory differences were found in the non-protein nitrogen of blood by precipitating the proteins with different acids (Sjollema, B., and Hetterschy, C. W. G., *Biochem. Z.*, 1917, lxxxiv, 371).

TABLE III.

Samples of Milk from Udders That Were Examined Microscopically.

No.	Acidity	Oxalate acidity.	Sediment.	Leucocytes.	CaO mg. per 100 cc.	Non-protein nitrogen. mg. per 100 cc.	Remarks.
359	10 9, after 24 hours.	6. 28, after 24 hours.	6 per cent with blood.	Too many to count.	272		Interstitial tissue of the udder greatly developed. Nearly all acini filled up with a homogeneous substance, which was evenly stained red by eosin. No symptoms of inflammation, no bacteria.
347a R.F.	6. 2		0 25 per cent.	More than normal.			Tissue, R.F. and R.H., almost normal. The lumina of the alveoli are wide. The tissue
347b R.H.	6 0		0 3 percent.				of the L.F. has many alveoli with pus, containing polymorphonuclear leucocytes. The nuclei strongly stained with hematum.
347c L.F.	2 9		0 5 cc.	Too many to count.	208		
302	2 4			Appearance nearly normal, a fair amount of epithelial cells, a small number of leucocytes.	295	40	No microorganisms. Chronic interstitial mastitis. Most glandular alveoli without content; some with a substance that is stained red with eosin. In the alveolar lumina a very small number of cells, no polymorphonuclear nor polymorphonuclear leucocytes. The interstitial area largely increased and with all sorts of cells char-

304	3.3	1.65		Coffee - colored, odor of putrefactive proesses, neither streptococci nor leucocytes.	103	68	Normal udder in involution. No bacteria.

tophane could be detected. The very high tryptophane figures
for colostrum can probably be explained by the high percentage
of globulins. We determined a few times the amount of trypto-
phane in normal milks and in blood plasma (of cows) without
treating them with potassium-aluminium sulfate or any other
protein-precipitating reagent. We found about 100 mg. in 100 cc.
of milk and about 400 to 600 mg. in blood plasma.

Our experience is not sufficient to state that at the end of the
lactation period tryptophane increases in the milk. We are
examining this point as well as others connected with the trypto-
phane question; i.e., in how far the identity of the proteins of
blood plasma and lymph with those of abnormal milks can be
examined by means of the estimation of tryptophane and
whether the amount of tryptophane is increased in abnormal milks
if the affection that influences the milk secretion is somewhere
else than in the udder.

In reference to the last point we refer to the data of Milk 230.
The samples of the different quarters gave normal tryptophane
figures. There was no udder disease to be found nor any other
disease; the milk yields were very low. They were abnormal with
regard to several points: acidity, chlorides, lactose, leucocytes,
and catalase. It may be considered to be of importance, that
we found leucocytes to be poor in tryptophane; therefore a
direct relation between the number of leucocytes and the amount
of tryptophane does not exist. We wished to know whether the
streptococci were retained by the udder tissues in those cases
in which milk of abnormal chemical composition contained no
streptococci. If so, the conclusion from the microscopic examina-
tion of the milk would have been incorrect. For this purpose we
analyzed the milk of animals brought to the abattoir to be slaught-
ered owing to serious udder diseases.

Mr. H. S. Frenkel kindly examined parts of the udder tissues
microscopically to find out whether streptococci were present
and to determine the nature of the mastitis. We cannot guar-
antee that the cows were in full lactation and regularly milked.
As seen from the data given in Table III we found that samples
of milk of a very abnormal composition were at the same time
devoid of streptococci. It is, of course, impossible to state, that
in these cases streptococci originally played no rôle. Two objec-

tions may be made. The first is that we made no cultures; *i.e.*, that we made no second microscopic examination after incubating the milk for some time at 37°C. We do not think it probable, that when streptococci cannot be detected, either in the milk or in the udder tissue, that they actually have a great influence on the milk secretion. The second objection may be that milk drawn at irregular times is not normal milk. Yet it is evident, that not all the abnormalities which the samples show, can be caused in that way; *i.e.*, Milk 347, a, b, and c drawn from different quarters differs greatly. An inflammation must have been the cause of this.

SUMMARY.

This paper shows that an aseptic inflammation of the udder, produced by an injection of a silver nitrate solution, causes the same changes of the milk as an infection with bacteria; *i.e.*, by streptococci. This renders uncertain the fact that streptococci usually originate the production of abnormal milk and of the inflammation of the udder.

An aseptic abscess produced in the region of the neck influenced the composition of the milk very little. The changes were not in the sense observed in udder diseases or in some other affections of the milch cow.

In some cases of serious udder diseases the udder tissues were devoid of streptococci. This holds good in cases in which the milk possessed the composition typical of streptococcic mastitis. Data are given of a number of samples of abnormal milk. The acidity of these samples was high, low, or normal. Independent of the acidity the amounts of chlorides, lactose, etc., were abnormal. Some other affections of the milch cow produce the same changes of the milk, except the increase of the number of leucocytes and the differences between the milk drawn from the four quarters.

Streptococci have little influence on the amount of non-protein nitrogen of the milk. *Pyogenes* increases this figure considerably.

The determination of tryptophane is one of the best reagents on milk of inflamed udders.

Colostrum serum contains much more tryptophane, for example 50 times as much, than normal milk serum.

An abnormally low acidity of milk can easily be detected with phenol red after adding potassium oxalate.

In milks with a very low acidity streptococci do not appear to be present.

BIBLIOGRAPHY.

1. Baker, J. C., and Breed, R. S., *J. Biol. Chem.*, 1920, xliii, 221.
2. Fürth, O., and Nobel, E., *Biochem. Z.*, 1920, cix, 103.
3. Baker, J. C., and Breed, R. S., *New York Agric. Exp. Station*, Circular 58.
4. Baker, J. C., and Van Slyke, L. L., *J. Biol. Chem.*, 1919, xl, 357.
5. Sjollema, B., *Compt. rend. Congr. Internat. de Pharm. La Hage*, 1913, 1052; *Handelingen Gen. u. Heilkunde*, 1910.
6. Bergema, R., *Jahrb. Milchwirtsch.*, 1915; Inaugural dissertation, Bern, 1914.

INDEX TO AUTHORS.

INDEX TO SUBJECTS.

THE JOURNAL

OF

BIOLOGICAL CHEMISTRY

VOLUME LIV
BALTIMORE
1922

CONTENTS OF VOLUME LIV.

No. 1. September, 1922.

Contents

No. 2. October, 1922.

Contents

No. 3. November, 1922.

Contents

NOTE ON THE RELIABILITY OF THE BENEDICT AND FOLIN-WU BLOOD SUGAR DETERMINATIONS.

By F. A. CSONKA and GRACE C. TAGGART.

(From the Laboratory of Dr. J. P. McKelvy, Pittsburgh.)

A comparative study of blood sugar methods was recently published in this Journal by Höst and Hatlehol;[1] their conclusion was that the Benedict[2] method gives a generally higher result than the Folin-Wu[3] method. Since then, Folin and Wu have modified their technique and claim that "the new process tends to give slightly lower values than the original method." This makes the difference between the Benedict and Folin-Wu methods even larger than the tables of Höst and Hatlehol show. Our aim at present is, not to prove whether the blood sugar values obtained by the above mentioned methods are absolutely correct or whether reducing substances other than glucose present in the blood have any influence on the reagents used, but to decide which of these two methods gives the more reliable results.

A 1 per cent solution of pure glucose was made up, using Kahlbaum's (c.p.) preparation, which, by dilution, gave the necessary standards for both methods and a third "unknown" solution was prepared containing approximately 20 mg. per 100 cc. We found that, using these pure glucose solutions, both methods gave identical and correct results. What, then, can be the reason for the different results obtained by the two methods when applied to human blood; *i.e.*, higher results according to Benedict's method? (We must keep in mind that Benedict eliminates the blood proteins by picric acid and Folin and Wu, by tungstic acid). In the first place, since a higher result (Benedict) indicates a larger amount of glucose present in the blood filtrate, we might

---

[1] Höst, H. F., and Hatlehol, R., *J. Biol. Chem.*, 1920, xlii, 347.

[2] Benedict, S. R., *J. Biol. Chem.*, 1918, xxxiv, 203.

[3] Folin, O., and Wu, H., *J. Biol. Chem.*, 1920, xli, 367.

1

conclude that the tungstic acid precipitation is responsible for the loss of some glucose. In the second place, we might conclude that there are certain reducing substances in the filtrates other than glucose which react with the picric acid but do not affect the copper.

To settle the first possibility we used the Folin-Wu technique on both the tungstic and the picric filtrates[4] and, to our satisfaction, found very good checks. This disproves the theory that the tungstic acid precipitation is responsible for the loss of glucose. To counteract the yellow color of the picric acid we added

TABLE I.

Blood Sugar in Mg. per 100 Cc. of Blood.*

Subject.	Condition.	Tungstate filtrate.		Picric-picrate filtrate.	
		Folin-Wu method.	Benedict technique.	Folin-Wu technique.	Benedict method.
		mg.	mg.	mg.	mg.
1	Normal.	73	86	72	120
2	"	83	82	81	117
3	"	87	100	86	121
4	"	88	91	87	118
5	"	89	94	87	120
6	Diabetic.	87	104	81	130
7	"	91	106	89	122
8	"	97	110	96	150
9	"	140	157	133	170
10	"	164	162	161	184
11	" accompanied by glycosuria.	200	230	200	263

* Blood was taken before breakfast.

1 to 1.2 cc. of the picric-picrate solution to the Folin standard before dilution; the green color produced in both standard and unknown could be matched with ease in the colorimeter. As seen readily from Table I, the results are within the limit of experimental error (see Column 5) and can be assumed as identical. At the same time this experiment serves as a proof that the copper is not reduced by the non-glucose substance which reacts with the picric acid and produces a higher figure for blood sugar (Benedict).

[4] It was found that the presence of picric acid did not influence the copper reduction.

It was of further interest to apply the tungstic acid filtrate to the Benedict technique (4 cc. of filtrate and 4 cc. of picric-picrate solution). As seen from Table I, Column 4, the results are sometimes higher than those of Folin but always lower than those of Benedict. This shows that the tungstic precipitation partially eliminates the non-glucose reducing material and could be advocated as an improved modification. To simplify calculations a standard solution of glucose containing 0.4 mg. per 4 cc. may be used.

In conclusion, we may state that the Folin-Wu blood sugar determination gives more reliable results than Benedict's picric-picrate method.

THE MINERAL CONTENT OF THE NORMAL WHITE RAT DURING GROWTH.

By G. DAVIS BUCKNER and A. M. PETER.

(From the Department of Chemistry, Agricultural Experiment Station, University of Kentucky, Lexington.)

The object of the experiment reported herein was to determine the mineral content of the carcasses of normal white rats at different ages. The laboratory conditions under which the animals were raised are reproducible. The use to be made of the data will be for comparison with similar data to be procured under a change of the minerals ingested.

A large number of healthy adult white rats were obtained which had come from common parents and had existed under identical conditions since weaning. These rats were bred and only those litters from which three males and three females could be selected for analysis were used in the experiment. These litters were weaned at the age of 4 weeks and at that time separated as to sex. After weaning, their principal food consisted of cracked yellow corn which was kept before them at all times. Three times a week stale light bread soaked in fresh whole milk was fed as the morning meal in such quantities as they would consume in 30 minutes and twice a week shredded cabbage was fed. Laboratory tap water was used throughout. They were bedded with clean oat straw which was changed twice a week.

When a group of rats reached the desired age they were chloroformed and thoroughly washed with distilled water and dried. The esophagus, stomach, intestines, and rectum were dissected out and discarded. The remaining portions of the three rats of each sex were weighed together and ashed at a dull red heat in a muffle for 12 hours, thereby obtaining a nearly white ash, very uniform in color and texture. After weighing, the composite sample of ash was well mixed by grinding and a weighed

5

portion dissolved in hydrochloric acid and analyzed for calcium and magnesium by the method of McCrudden.[1]   Phosphorus and potassium were determined by the methods of the Association of Official Agricultural Chemists[2] after solution in hydrochloric acid.

The results are shown in Tables I and III.   Tables II and IV were computed from the data in Tables I and III.

It will be seen that the weights of the male and female carcasses increase regularly for 40 weeks, except between the 16th and 18th weeks for the males and the 10th and 12th and the 14th and 16th weeks for the females.   In these cases the deficiency in weight was due, perhaps, to individual variations.

The weights of the carcasses of the males and females of the same age up to 10 weeks are comparable but after that age the males are appreciably heavier than the females.

With the exception of the percentages of ash in the carcasses of the 2 week old male and the 20, 30, and 40 week old males and females, the percentage remains fairly constant in the intervening periods.   The percentage of P_2O_5 in the ash of the carcass shows a wider range of variation in the female than in the male but the maximum in the male is higher.   The total weights of P_2O_5 in the carcass of both males and females show an increase in each period except that of 18 weeks for the former and of 12 weeks for the latter.   Here again we have cases of individual variation.

The percentages of CaO in the ashes of the males vary more than those of the females, but the average is about the same.   The weights of CaO in the 18 week old male and the 12 week old female are smaller than those for the corresponding weights for the 2 weeks previous.

It is interesting to note that at the age of 2 weeks the oxygen ratio of $CaO + MgO$ to P_2O_5 approximates that of tricalcium phosphate (1.67), whereas, for the older rats this ratio falls between that and the ratio for dicalcium phosphate (2.5); and that the ratio of calcium to magnesium and to potassium is sensibly the same for both sexes and all ages.

---

[1] McCrudden, F. H., *J. Biol. Chem.*, 1909–10, vii, 83.

[2] Official and tentative methods of analysis of the Association of Official Agricultural Chemists, revised, November 1, 1919, pp. 3 and 12.

TABLE I.

Average Weights per Carcass, Grams, and Percentage of Ash.

Age.	Carcass without alimentary canal.		Crude ash.		Percentage of ash.	
	Male.	Female.	Male.	Female.	Male.	Female.
wks.						
2	15 65	12.27	0.28	0.34	1 79	2.77
4	17.76	15.91	0.52	0.47	2.93	2 95
6	20 80	21.20	0.62	0 65	2 98	3.07
8	45 01	47.32	1.47	1.41	3.27	2.98
10	Lost.	63.34		1.84		2.90
12	60.02	53.85	1.70	1 55	2.82	2.88
14	93.66	79.07	2.40	2.19	2.56	2.77
16	111.21	68.19	3.49	2.10	3 14	3.08
18	102 09	93.19	3.04	2.89	2.98	3.10
20	124.55	100.64	3.29	3.36	2 64	3.34
30	160.21	142.35	5.79	4.80	3.61	3 37
40	180.50	138.81	7.14	5.37	3.95	3.87
Average............	84 68	69.68	2.70	2.24	2.95	3.00
Gain in 38 weeks..	174.85	130.08	6.86	5 03	2.16	1.10

TABLE II.

Average Percentages of Mineral Substances in the Carcasses.

Age.	P₂O₅		CaO		MgO		K₂O	
	Male.	Female.	Male.	Female.	Male.	Female.	Male.	Female.
wks.								
2	0.66	0 87	0.72	0.99	0 04	0 06	Lost.	Lost.
4	1.28	1.27	1.03	1 05	0.07	0 07	0.04	0.04
6	1.35	1.32	1.12	1.09	0.07	0 07	0.03	0.04
8	1.40	1.23	1.12	1.00	0 08	0 07	0.04	0 03
10		1.23		1 04		0.07		0 03
12	1.18	1.12	1.02	0.99	0 04	0 06	0.03	0.04
14	1.07	Lost.	0.99	1 00	0 06	0.06	0 03	0.03
16	1.33	1.24	1.14	1.11	0 07	0.07	0 04	0.03
18	1.22	1.28	0 99	1 09	0 06	0 07	0.03	0.03
20	1.07	1 41	0 89	1.22	0 06	0 08	0.03	0.04
30	1.42	1.35	1.31	1.20	0 07	0.07	0 03	0.04
40	1.79	1.51	1.47	1.41	0 09	0.08	0.04	0 03
Average..	1.25	1 26	1 07	1 10	0 06	0 07	0 04	0 03

TABLE III.

Percentage Composition of the Crude Ash.

Age.	P_2O_5		CaO		MgO		K_2O		Undetermined.	
	Male.	Female.	Male.	Female.	Male.	Female.	Male.	Female.	Male.	Female.
wks.										
2	37.07	31.53	40.24	35.30	1.99	2.16	Lost.	Lost.	20.70	31.01
4	43.85	43.00	35.25	35.56	2.30	2.28	1.50	1.31	17.10	17.85
6	45.40	43.10	37.50	35.40	2.26	2.20	1.13	1.18	13.71	18.12
8	42.90	41.40	34.10	33.40	2.35	2.47	1.19	1.10	19.46	21.63
10		42.41		35.72		2.44		1.13		18.30
12	42.00	39.00	36.00	34.20	2.19	2.19	1.06	1.55	18.75	23.06
14	41.85	Lost.	38.65	34.39	2.29	2.07	1.20	1.21	16.01	
16	42.31	40.10	36.36	36.10	2.12	2.19	1.15	1.13	18.06	20.48
18	41.10	41.20	33.30	35.30	2.06	2.35	1.03	1.03	22.51	20.12
20	40.60	42.20	33.70	36.50	2.37	2.25	1.00	1.10	22.33	17.95
30	39.40	39.95	36.20	35.67	1.97	2.11	0.80	0.92	21.63	21.35
40	45.40	39.70	37.20	36.40	2.31	1.98	1.01	0.86	14.08	21.06
Average..	41.99	40.32	36.23	35.33	2.20	2.22	1.11	1.14	18.58	20.99
Range....	8.33	11.57	6.94	3.10	0.40	0.49	0.70	0.69	8.80	13.16

TABLE IV.

Oxygen Ratios.

Age.	$\dfrac{\text{O of } P_2O_5}{\text{O of CaO} + \text{MgO}}$		$\dfrac{\text{O of CaO}}{\text{O of MgO}}$	
	Male.	Female.	Male.	Female.
wks.				
2	1.70	1.62	11.5	11.7
4	2.24	2.19	11.1	11.3
6	2.20	2.20	11.9	11.6
8	2.27	2.22	10.5	9.7
10		2.14		10.5
12	2.12	2.07	11.8	11.2
14	1.98		12.1	11.4
16	2.12	2.02	12.4	11.8
18	2.23	2.11	11.6	10.8
20	2.17	2.10	10.2	11.7
30	2.00	2.04	13.2	12.1
40	2.22	2.00	11.5	13.2
Average.....	2.11	2.07	11.9	11.5

These figures show that rats coming from a common parent stock, having been similarly fed in all cases and all having existed under identical conditions have an individual variation in growth and mineral content in both males and females which is not overcome when results are compiled from the average of three of each sex.

A STUDY OF THE DISTRIBUTION OF IODINE BETWEEN CELLS AND COLLOID IN THE THYROID GLAND.

IV. THE DISTRIBUTION OF IODINE IN THE HYPERPLASTIC THYROID GLAND OF THE DOG AFTER THE INTRAVENOUS INJECTION OF IODINE COMPOUNDS.

By HARRY BENJAMIN van DYKE.

(*From the Laboratories of Pharmacology, University of Chicago, Chicago.*)

Some years ago Marine and Feiss (1) and Marine and Rogoff (2, 3) first performed experiments which leave little doubt as to the ability of the dog's thyroid gland, especially when hyperplastic, to bind iodine almost instantaneously. Marine and Feiss (1) carefully perfused the surviving thyroid gland with fluid containing iodine as KI. They found that after 1 hour a considerable amount of iodine was taken up only by a *surviving* gland. Any evidence of death of the perfused organ was accompanied by a loss of some of the gland's stored iodine rather than by an absorption of iodine from the circulating medium. However, even a surviving gland, rich in iodine, lost some iodine to a perfusing fluid free of the element. In surviving glands they discovered that the amounts of iodine absorbed were relatively independent of the amounts of iodine in the perfusing fluids. They also pointed out that a similarly rapid absorption of iodine by the intact gland follows the intravenous administration of a solution of KI. From the results of perfusions of spleen and kidney under similar conditions they concluded that these organs were not capable of taking up a significant amount of iodine.

Marine and Rogoff (2) on the basis of experiments in which they injected a solution of KI intravenously came to the conclusion that the absorption of iodine by the gland is almost as great 1 hour after the injection as it is 30 hours after the injection. They again found that no significant amount of iodine was taken up by the spleen and liver. The amount of iodine absorbed by the

thyroid gland appeared to be directly proportional to the degree of hyperplasia exhibited by the gland. In a second communication (3) they discussed the time of appearance of the changes in the histology and physiological activity of the gland following the intravenous administration of KI solution.

Having found (4) that there was relatively little difference in the ratio of the percentage of iodine in cells to the percentage of iodine in whole gland in dog thyroid glands exhibiting great variations in histological appearance and iodine content, I undertook the present study to determine what effect acute iodization of hyperplastic thyroid glands has on the ratio value.

Methods.

As in the work of Marine and Feiss, and Marine and Rogoff, dogs with thyroid glands usually definitely hyperplastic were used in all of the experiments. Light ether narcosis was always employed. All solutions of KI and thyroid colloid were injected into the femoral vein. Every effort was made to section the glands as rapidly as possible after their removal from the animal. In the making of the determinations of the ratio of the percentage of iodine in dried cells to that in dried whole gland the method first described by Tatum (5) was somewhat modified. To lessen autolysis as much as possible the glands on removal were plunged into Ringer's solution cooled to 1–4°C. The glands were then carefully cut into blocks of a size appropriate for the floor of the freezing microtome and frozen sections were made of a considerable amount of tissue. The blocks of tissue cut for the microtome's floor as well as the cut sections were suspended in Ringer's solution cooled to 1–4°C. As in the preceding work of this series the frozen sections were cut sufficiently thin so that practically all of the colloid or intercellular fluid was dissolved out of the acini as soon as the sections were suspended in cooled Ringer's solution. By centrifugalization the cells were separated from the colloid dissolved in the Ringer's solution and carefully dried over an electric hotplate and then in an electric drying oven. Control pieces of uncut whole gland were also dried in the same manner. Throughout this paper colloid iodine solutions refer to cell-free solutions of thyroid intercellular fluids obtained by the method just described and dissolved in Ringer's solution. In all of the experiments the

quantitative determinations of iodine were made by the method of Kendall (6).

From the data of Table I it can be seen that large amounts of iodine as KI were taken up by hyperplastic thyroid glands despite big variations in the dose of KI (50 to 150 mg.) and duration of time between injection and removal of gland (90 seconds to 22 hours and 25 minutes). The cooling of the blocks of thyroid tissue during the process of cutting had little effect. Either little of the injected iodine was held by the cells, or it diffused rapidly from the cells in the process of cutting; for the iodine content of the cells and hence the ratio of the precentage of iodine in cells to the percentage of iodine in whole gland is considerably less than that found in resting glands. The average ratio value of 0.15 for the dog (4) was approached only in Experiment 11 in which nearly 24 hours elapsed between the injection of KI and the removal of the experimental lobe. In this last case there are alternative means of explanation: more iodine may have been bound by the cells by removal from the thyroid's intercellular spaces, or the iodine in the cells was in a less diffusible form than in the other experiments of the series. These changes in the distribution of iodine after about 24 hours can be definitely correlated with Marine and Rogoff's (3) discovery that 20 hours after the injection of iodine as KI, markedly hyperplastic glands exhibit more stainable colloid with some increase in the size of the follicular spaces and shrinkage in the height of the columnar epithelium.

In the experiments the data of which are given in Table II, solutions of colloid iodine from normal dog thyroid glands were injected intravenously to determine to what extent iodine in organic combination is bound by hyperplastic thyroid glands.

The iodine of thyroid colloid of normal animals is practically not at all taken up by hyperplastic glands after 42 to 86 minutes. A comparable amount of iodine in the form of KI is rapidly taken up.

Blum and Grützner (7) found some hours after the injection into the circulatory system of fluid pressed from thyroid glands that the iodine-containing thyroid protein is split largely by hepatic action into simpler products with the formation even of iodides. In Experiments 12 and 13 (42 and 86 minutes after intravenous injection) there is little evidence that much iodide-

Animal No.	Amount of KI injected.	Time elapsing between injection and removal of gland.	Lobe.	Weight of whole gland used.	Iodine in whole gland.	Weight of cell mass used.	Iodine in cell mass.	Ratio of per cent of iodine in cells to per cent of iodine in whole gland.
	mg.	*sec.*		*gm.*	*per cent*	*gm.*	*per cent*	
1	50	90	Control.	0.6786	0.021	0.5242	0	
			Iodized.	0.5553	0.042	0.7845	0.002	0.048
2	50	150	Control.	0.6548	0.010	0.6373	0	
			Iodized.	0.5695	0.072	0.8437	0.003	0.042
		min.						
3*	135	57	Control.	0.6797	0	0.3585	0	
			Iodized.	0.4808	0.086	0.5449	0	
4	120	60	Control.	0.4512	0.042	0.3775	0	
			Iodized.	0.7760	0.079	0.4702	0	
5	150	60	Control.	0.6853	0	0.6105	0	
			Iodized.	0.6302	0.078	0.7473	0	
6	50	60	Control.	0.8779	0.002	0.5694	0	
			Iodized.	0.8314	0.059	0.8021	0.004	0.068
7	50	60	Control.	0.8130	0			
			Iodized.	0.6521	0.085	0.6994	0.002	0.025
				0.7311	0.070			
8	50	60	Control.	0.4082	0.007			
				0.6490	0.004			
			Iodized.	0.4610	0.081	0.8222	0.004	0.045
				0.2455	0.098			
9	150	64	Control.	0.5150	0.002	0.7066	0	
			Iodized.	0.5957	0.085	0.8282	0.003	0.035
10*	120	70	Control.	0.3458	0.010	0.2459	0	
			Iodized.	0.2502	0.091	0.2627	0	
		hrs. min.						
11	50	22 25	Control.	0.4650	0.017	0.5357	0	
				0.5715	0.020			
			Iodized.	0.7191	0.213	0.5422	0.022	0.103

* Glands and cells not suspended in ice-cooled Ringer's solution.

14

iodine has been split from colloid iodine; for the hyperplastic thyroid glands present exhibit no significant change in iodine content and yet are able quickly to bind any iodine available as iodide.

In the experiments reported in Table III colloid iodine solution of animals with hyperplastic glands, each of which had received an

TABLE II.

Ratio of the Percentage of Iodine in Cells to the Percentage of Iodine in Whole Gland in Hyperplastic Thyroid Glands of Dogs after the Intravenous Injection of Thyroid Colloid Iodine from Normal Dog Thyroid Glands. The Iodine Content of a Hyperplastic Gland of a Dog after the Injection of a Comparable Amount of Inorganic Iodine is also Recorded.

Animal No.	Amount of solution injected.	Form of iodine.	Amount of iodine.	Time elapsing between completion of injection and removal of iodized lobe.	Lobe.	Weight of whole gland used.	Iodine in whole gland.	Weight of cell mass used.	Iodine in cell mass.	Ratio of per cent of iodine in cells to per cent of iodine in whole gland.
	cc.		mg.	min.		gm.	per cent	gm.	per cent	
12	200 0	Colloid iodine.	1.690	42	Control.	0.6565	0.002	0.5478	0.001	
					Iodized.	0.6248	0.002	0.4819	0	
13	259.6	Colloid iodine.	2.829	86	Control.	0.7971	0	0.7698	0	
						0.8557	0			
					Iodized.	0.4742	0 005	0.9208	0	
						0.4245	0			
14	241.5	KI	2.087	46	Control.	0 7939	0.003			
						0.6024	0.004			
					Iodized.	0 4513	0.017			
						0.5907	0.019			

intravenous injection of 50 mg. of KI 60 minutes before the removal of the thyroid lobe for section, was injected intravenously as soon as possible into other animals with hyperplastic glands. The sequence was as follows: as a control, part of one lobe was removed; colloid iodine dissolved in Ringer's solution was then injected and all but half of one lobe resected; a comparable amount of iodine as KI dissolved in a similar amount of Ringer's solution was then

TABLE III.

Ratio of the Percentage of Iodine in Cells to the Percentage of Iodine in Whole Gland in Hyperplastic Thyroid Glands of Dogs after the Intravenous Injection of Thyroid Colloid Iodine from Hyperplastic Thyroid Glands Iodized but a Short Time before Being Sectioned.

Animal No.	Form and amount of iodine injected.	Time elapsing between injection and removal of lobe sectioned or analyzed.	Lobe.	Weight of whole gland used.	Iodine in whole gland.	Weight of cell mass used.	Iodine in cell mass.	Ratio of per cent of iodine in cells to per cent of iodine in whole gland.
		min.		gm.	per cent	gm.	per cent	
15	0 820 mg. of colloid iodine of Animal 7* dissolved in 233 cc. of Ringer's solution.	60	Control. Colloid iodine.	0.6022 0.6986 0.7186	Trace. 0.009 0.009	1.1376	0	
	0 836 mg. of KI dissolved in 253.5 cc. of Ringer's solution.	25	KI	0 8328 0.6935	0.017 0 022			
16	0.903 mg. of colloid iodine of Animal 8* dissolved in 318 cc. of Ringer's solution.	40	Control. Colloid. iodine.	0.4700 0.4015 0.3695	0 0 006 0.007	1.1110	0	
	0 827 mg. of KI dissolved in 318 cc. of Ringer's solution.	6	KI	0.4581 0.4437	0 009 0.010			

* See Table I.

injected and the remaining half of one lobe taken out. Much more of the iodine from the colloid iodine of these animals was taken up than from the colloid iodine of normal animals (Table II).

Even here considerable iodine as KI was still bound by the glands which had taken up some colloid iodine. These results suggest that the colloid iodine, bound but incompletely synthesized into active principle, is in a more diffusible form in the acutely iodized gland than in the more normal resting gland. Moreover, the incompletely synthesized active principle may be more readily split by hepatic action (7) into simpler products (*e.g.*, iodides) which are then bound by the hyperplastic thyroid gland.

Marine and Rogoff (3) declare that even 30 hours after intravenous administration of a solution of KI very little of the thyroid's active principle, as measured by the effects of thyroid on tadpole growth and metamorphosis, has been elaborated. The less diffusible and less readily split normal colloid iodine compound (Table II) probably represents the more fully elaborated active principle.

SUMMARY.

The findings of Marine and Feiss and Marine and Rogoff that the hyperplastic thyroid gland of the dog rapidly binds iodine intravenously introduced as a solution of KI were confirmed.

By a method already described (5) the ratio value of iodine in cells to iodine in whole gland was determined and found to be very low after the intravenous injection of KI solution into dogs with hyperplastic glands when those glands were removed 1.5 to 60 minutes after the injection. The ratio value more nearly approached the normal if the interval elapsing between injection and removal of gland was made about 24 hours instead of 1 hour or less as in most of the experiments. This finding is in keeping with the histological changes described by Marine and Rogoff in such glands 20 hours after the intravenous injection of KI solution.

When iodine as colloid iodine solution of normal animals was administered intravenously practically none of the colloid iodine was taken up by hyperplastic glands during the periods of time used in these experiments; yet from an injection of a comparable amount of iodine in the form of KI the ready binding of iodine by similarly hyperplastic glands was proved. Colloid iodine of hyperplastic glands removed 1 hour after the intravenous injection of KI solution was taken up to some extent by hyperplastic glands; but these last named glands bound additional iodine as KI intro-

duced after the colloid iodine injection. The incompletely synthesized active principle is probably more diffusible and more readily split into simpler products than active principle fully synthesized.

BIBLIOGRAPHY.

1. Marine, D., and Feiss, H. O., *J. Pharmacol. and Exp. Therap.*, 1915, vii, 557.
2. Marine, D., and Rogoff, J. M., *J. Pharmacol. and Exp. Therap.*, 1916, viii, 439.
3. Marine, D., and Rogoff, J. M., *J. Pharmacol. and Exp. Therap.*, 1916–17, ix, 1.
4. van Dyke, H. B., *J. Biol. Chem.*, 1920–21, xlv, 325.
5. Tatum, A. L., *J. Biol. Chem.*, 1920, xlii, 47.
6. Kendall, E. C., *J. Biol. Chem.*, 1914, xix, 251.
7. Blum, F., and Grützner, R., *Z. physiol. Chem.*, 1920, cx, 277.

THE FERMENTATION OF PENTOSES BY MOLDS.*

By W. H. PETERSON, E. B. FRED, and E. G. SCHMIDT.

(From the Departments of Agricultural Chemistry and Agricultural Bacteriology, University of Wisconsin, Madison.)

In the numerous studies which have been carried out on the nutritive value of different carbon compounds for molds the forms most generally used have been *Aspergillus* and *Penicillium*. It has been found that these fungi can ferment carbohydrates, alcohols, organic acids, fats, proteins, and many other substances; but the fermentation of the pentoses and pentose-yielding substances has been investigated to only a limited extent. The general distribution of pentose-yielding substances in nature, and the rapidity with which such compounds must be broken down suggests the possibility that fungi play an important rôle in their destruction.

The literature on the subject of carbon utilization by molds is far too voluminous to review in this paper. Only such work as deals specifically with the fermentation of the pentoses and the general products of mold metabolism will be considered. For an extensive review of the literature concerning the growth and carbon assimilation of the molds, the reader is referred to the papers of Lafar (1) and Steinberg (2).

The assimilation of carbon from arabinose and xylose by various fungi has been mentioned by a few investigators. Behrens (3) in 1898 was perhaps the first one to note the favorable effect of arabinose on the growth of *Penicillium glaucum* and *Botrytis vulgaris*. In 1901 Went (4) found that xylose serves as a good source while arabinose is a poor source of carbon for *Monilia sitophila*

(*Mont.*) Sacc. 2 years later Emmerling (5) found that *Aspergillus niger* assimilates arabinose and xylose, but does not form oxalic acid from these compounds. In 1913 Krüger (6) showed that a certain species of *Glocosporium* can utilize arabinose. Hawkins (7) in 1915 carried out a study of the utilization of pentosan and pentoses by *Glomerella cingulata*. He concluded that the pentoses are readily utilized by this fungus; in fact, arabinose and xylose seemed better sources of carbon than glucose. From this brief review it is evident that but little attention has been paid to the utilization of the pentoses, the products formed, and the relation of these products to one another and to the sugar consumed.

As a means of measuring mold activity one or more of the following indices have been used by most investigators: weight of mycelium formed, carbon dioxide produced, or sugar consumed. Of these three the first is the one most commonly used. The weight of mycelium is greatly influenced by the conditions of growth and the kind of fungus, but under normal conditions the common molds consume about 3 gm. of sugar in the production of 1 gm. of dry fungus. Weimer and Harter (8), however, report an extreme case where 29 gm. of glucose were required to produce 1 gm. of fungus. Since the elementary composition of dry fungus and the compound fermented are different, Waterman (9) compared the two on the basis of their carbon content. From the common sugars he recovered about 35 per cent of the carbon in the mold pad. He termed this percentage the "plastic equivalent" of the fermented compound.

Although carbon dioxide is generally recognized as the chief fermentation product formed by fungi, comparatively few determinations of the quantity of gas evolved are found in the literature (10). Expressed as the weight of carbon dioxide to weight of fungus, respiratory coefficient, a ratio of approximately 2 has been found by a number of investigators. Of the carbon contained in the fermented compound from 50 to 70 per cent, depending on the fungus and the conditions of growth, are recovered in the carbon dioxide.

In the experimental part of this paper the destruction of sugar has been used as a guide in the selection of pentose-fermenting molds. The molds thus selected have been used for a more extended study of mycelian growth and carbon dioxide evolution.

TABLE I.

Rate of Fermentation of Arabinose and Xylose by Various Molds.

Medium contained 2 per cent of sugar.

Culture No.	Name	Sugar present after							
		73 hrs.		93 hrs.		142 hrs.		309 hrs.	
		Arabinose.	Xylose.	Arabinose.	Xylose.	Arabinose.	Xylose.	Arabinose.	Xylose.
		per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent
10	A. flavus.	0 04	0.70	0.00	0.09				
14	" "	0.21	1.19	0 00	0.05				
15	" "	0.87	0.29	0.00	0.00				
16	" fumigatus.	1.20	0.77	0.92	0.14	0.00	0.00		
17	" "	1.60		1.05	0.83		0.00		
13	" globosus v.				1.90	1.95	1.71	1.72	1.02
5	" niger.	0.00	0.00						
11	" "	0.00	0.25		0.00				
24	" oryzæ.			0.84	1.56	0.00	0.00		
12	" repens.			1.35	1.72	0.00	1.59		0.19
1	" sp.	1.03	0.82	1.00	0.00	0.06			
4	" "	0.37	0.00	0.00					
21	" "	1.33	1.13	1.19	0 38	0.00	0.00		
22	" "	0.67	1.25	0.30	0.30	0.00	0.00		
26	P. camemberti.					1.86	1.80	1.85	1.70
6	" glaucum.	0.80	0.80	0.50	0.00	0.00			
7	" " I.		1.40	1.54		0.97	0.28	0.00	
25	" roqueforti.					1.89	1.68	1.85	0.36
18	" sp.				1.61	1.52	1.15	0.40	0.00
20	" "				1.53	1.82	1.33	0.93	0.00
23	" "				1.61	1.82	1.05	1.29	0.00
2	" "	1.65	1.16	1.61		1.49	0.00	0.07	
19	" "	1.63		1.58	1.82	1.37	1.56	0.77	0.61
8	Rhizopus nigricans.					1.80	1.80	1.80	1.70
9	Cunninghamella sp.					1.80		Lost.	1.20

The molds used in this work were from laboratory stock cultures or were secured from E. M. Gilbert of the Botany Department, from Miss Audrey Richards of the Forest Products Laboratory, and from Parke, Davis and Company. Altogether 25 cultures were studied representing *Aspergillus*, *Penicillium*, and *Mucor* forms. For many helpful suggestions the authors are indebted to J. B. Overton and to E. M. Gilbert of the Department of Botany, University of Wisconsin.

After many kinds of nutrient solutions were tried, the one recommended by J. B. Overton was found most satisfactory. Except in the study of citric acid production, this nutrient solution was used in all fermentations. The composition of the Overton medium is as follows:

	cc.
M ammonium nitrate	250
M monobasic potassium phosphate	100
M magnesium sulfate	40
M ferric chloride	2
Distilled water	608

The individual salts were made up in quantities sufficient to cover a series of experiments. Each stock salt solution was kept in a separate flask and was combined with the others as needed.

Sugar was dissolved in water to yield a 4 per cent solution. In the preliminary experiments, 100 cc. Erlenmeyer flasks were used. 10 cc. of the salt solution were placed in each flask which was then plugged with cotton and sterilized for 1 hour under 20 pounds of steam. The sugar solution was sterilized in a similar manner, and 10 cc. were transferred with a sterile pipette into each of the flasks, making 20 cc. of a 2 per cent sugar solution. These culture solutions were about 1 cm. deep. The cultures were inoculated with the mold spores and incubated at 28°C. At various intervals observations of the growth of the fungi and determinations of the unfermented sugar were made.

The Rate of Fermentation of Arabinose and Xylose by Various Molds.

A list of the molds[1] and the percentages of arabinose and xylose that remained unfermented at the end of certain intervals of time

[1] The term molds is used to include *Aspergillus*, *Penicillium*, and *Mucor*.

are given in Table I. Of the 25 molds tested, 16 made a fair to rapid growth, 9 grew slowly, and 3 or 4 of these produced only a few mycelian threads. The *Aspergillus niger* strains are especially active in the destruction of the pentoses. With the exception of *Penieillium glaucum* the *Penicillia* break down the pentoses only slowly and in the case of certain species are almost inactive. The two *Mueors* showed but little power to ferment the pentoses.

The two pentoses are apparently equally available to the molds. With the slow fermenting molds, xylose appears to be somewhat more rapidly utilized than arabinose.

A Comparison of the Rate of Fermentation of Xylose, Arabinose, and Glucose.

In this series it was planned to compare the fermentability of the pentoses with a well known hexose. It was felt that possibly some of the molds would break down the pentoses faster than glucose. This condition has been found to obtain with certain bacteria which are characteristically pentose-fermenters (11). Only the most vigorous of the molds were used in this series.

In order to give a longer time for comparison a 4 per cent solution of the sugars was used in each case, and the cultures were analyzed for sugar as soon as growth was apparent. To follow closely the rate of fermentation frequent analyses were run. The results of of these analyses are given in Table II.

An examination of the data in Table II shows that the destruction of glucose was more rapid than that of either of the pentoses. In one case, Culture 21, xylose seems to have disappeared more rapidly than glucose. Of the two pentoses, arabinose appeared to be slightly superior for the first period, 48 hours, but at the end of 70 hours the xylose cultures were ahead in every case. This superiority continues throughout all of the subsequent analyses until the end of the fermentation period. Some of the molds proved to be slow fermenters of the pentoses, especially of arabinose. Even at the end of 8 to 10 days there was still 1 per cent of sugar unfermented.

TABLE II.

A Comparison of the Rate of Fermentation of Xylose, Arabinose, and Glucose by Various Molds.

Medium contained 4 per cent of sugar.

Culture No.	Name.	Sugar fermented.	Sugar present after						
			48 hrs.	70 hrs.	95 hrs.	119 hrs.	143 hrs.	167 hrs.	215 hrs.
			per cent	per cent	per cent	per cent	per cent	per cent	per cent
15	A. flavus.	Xylose.	3.91	2.15	0.33	0.00			
	" "	Arabinose.	3.14	2.87	2.06		0.07	0.00	
	" "	Glucose.	3.14	0.96	0.03	0.00			
16	" fumigatus.	Xylose.	3.47	2.25	0.40	0.00			
	" "	Arabinose.	3.26	3.20	2.92		1.64	0.85	
	" "	Glucose.	3.15	1.69	0.29	0.00			
5	" niger.	Xylose.	3.48	0.13	0.00				
	" "	Arabinose.	2.87	1.28	0.00				
	" "	Glucose.	3.14	0.04	0.00				
11	" "	Xylose.	3.45	0.53	0.00				
	" "	Arabinose.	2.94	1.31	0.03	0.00			
	" "	Glucose.	2.94	0.16	0.00				
24	" oryzae.	Xylose.			3.56	3.08			0.00
	" "	Arabinose.		2.95	2.84		1.89	1.77	0.57
	" "	Glucose.			1.65	0.36	0.00		
12	" repens.	Xylose.					3.53	1.92	1.19
	" "	Arabinose.			3.35		2.12	2.04	2.00
	" "	Glucose.					2.95	1.71	0.00
21	" sp.	Xylose.			3.10		0.90		0.00
	" "	Arabinose.		3.33	3.19		1.87	1.34	0.00
	" "	Glucose.			3.47		1.70		0.00
6	P. glaucum.	Xylose.		2.81	1.41	0.09	0.00		
	" "	Arabinose.	3.13	2.80	2.50		1.43	0.70	0.00
	" "	Glucose.		2.26	0.90	0.00			

Weight of Mold Growth Produced from Xylose.

2 per cent of xylose in the nutrient solution was placed in Erlenmeyer flasks and inoculated with various molds. In one series, Cultures 1, 2, and 4, 50 cc. of medium in 150 cc. flasks were used, and in another series, Cultures 5, 6, and 7, 100 cc. in 300 cc. flasks. After practically all of the sugar had been destroyed, the pads of mold growth were carefully removed from the flasks, placed on a filter, and washed with water to remove adhering salts. The pads were dried in the oven at 100°C. and weighed. The data are given in Table III. The growth of *Penicillium glaucum* is less profuse than that of *Aspergillus niger*. This is evident to the naked eye, and it is also indicated by the dry weight of the pads.

TABLE III.

Relation of Mold Growth to Xylose Consumed.

Medium contained 2 per cent of sugar.

Culture No.	Name.	Age.	Weight of pad.	Economic coefficient.
		days	*gm.*	
1	A. sp.	9	0.778	2.56
2	" "	9	0.519	3.85
4	" niger.	9	0.918	2.18
5	" "	9	0.763	2.62
6	P. glaucum.	10	0.548	3.65
7	" "	11	0.620	3.22

The fermented solutions were tested for acidity, but no titratable acid was found, nor was any decided change found in the hydrogen ion concentration of the solution.

Relation of Carbon Dioxide and Mold Growth to Sugar Consumed.

In this experiment the quantity of carbon dioxide and the weight of the mold pad were determined. The fermentation was conducted in a closed system through which a slow current of carbon dioxide-free air was drawn. The carbon dioxide evolved by the molds was absorbed in 30 per cent potassium hydroxide and determined by double titration according to the method of Scales (12).

As a check on the Scales method, CO_2 was also determined by the gasometric method of Van Slyke (13). In the first experiment each fermentation flask contained 100 cc. of a 2 per cent xylose-salt solution. Three molds, Nos. 1, 5, and 6, were used in this fermentation, which was carried out in a warm room at about 25°C. Good growth was obtained in all the flasks. After 4 days the surface was well covered, and in the case of No. 5 black spores began to appear. After 14 days the train was taken down, the pads were removed, washed, dried, and weighed. The data are given in Table IV.

If the carbon in the carbon dioxide is compared with that contained in the xylose, it will be found that 62, 59, and 60 per cent, respectively, of the carbon in the xylose has been converted into

TABLE IV.

Relation of Carbon Dioxide Production and Weight of Mold Growth to Xylose Fermented.

Experiment No.	Culture No.	Name.	Age of culture.	Xylose fermented.	Carbon dioxide.	Weight of pad.	Respiratory coefficient.	Economic coefficient.
			days	gm.	gm.	gm.		
1	1	A. sp.	14	2.00	1.806	0.564	3.2	3.6
2	5	" niger.	14	2.00	1.716	0.690	2.5	2.90
3	6	P. gloucum.	14	1.47	1.288	0.400	3.2	3.77

carbon dioxide. This percentage relation was designated by Waterman as the respiratory equivalent, and was shown by him to vary with the age of the mold. In the early stages a large proportion of the carbon is stored in the pad. After all the sugar has been consumed, the cells draw upon that stored in the mycelian growth until they become almost inactive.

Rate of Carbon Dioxide Production and Distribution of Carbon in the Products of Fermentation.

Fermentation by Aspergillus niger, No. 5.—A 1,000 cc. Erlenmeyer flask with 2 per cent of xylose dissolved in the medium was used in these experiments. Before the gas was passed into the alkali solution, it was drawn through concentrated sulfuric acid in order to remove any traces of alcohol that might be produced. Instead of potassium hydroxide, a 0.4 N solution of barium hydrox-

ide was used as the absorbent. At approximately daily intervals this receiving bottle was removed, and the absorbed CO_2 determined by titration. From the data in Table V it is seen that evolution of gas begins after 24 hours and increases rapidly until a maximum is reached and then gradually declines. The maximum CO_2 production is reached within 3 or 4 days after the mycelium covers the surface of the liquid.

The percentage of the total carbon of the xylose represented by the CO_2 varies somewhat. Where all the sugar has been destroyed, it amounts to 41 per cent in Experiment 4, and 42 per cent in

TABLE V.

Evolution of Carbon Dioxide from Xylose by Aspergillus niger, No. 5.

Experiment 4.				Experiment 5.			
Age of culture.	CO₂ evolved.		Sugar fermented.	Age of culture.	CO₂ evolved.		Sugar fermented.
	Rate.	Total.			Rate.	Total.	
hrs.	*gm.*	*gm.*	*gm.*	*hrs.*	*gm.*	*gm.*	*gm.*
24	0.004			69	0.060	0.060	
48	0.050	0.054		90	0.332	0.392	
72	0.300	0.354		114	0 339	0.731	
76	0.106	0.460		144	0.500	1.231	
94	0.405	0.865		185	0.733	1.964	3.21
120	0.652	1.517	2.46	280	0.364	2.328	
148	0.312	1.829		405	0.150	2.478	
172	0.250	2.079	3.42	671	0.093	2.571	3.21
Weight of pad.........1.430 gm.				Weight of pad..........1.034 gm.			

Experiment 5. When CO_2 evolution has practically ceased as in Experiment 5, the carbon represented by the gas was 55 per cent of the total carbon of the xylose.

During the fermentation the sulfuric acid through which the gas was passed became gradually darker in color until at the end of the fermentation it was almost black. After oxidation with potassium dichromate the solution was distilled, but no volatile acid was obtained and hence no alcohol had been absorbed during the aspiration. The discoloration of the acid was due to some volatile organic substance. It is not improbable that the substance which produces the well known odor, so characteristic of molds, was the cause of the discoloration.

The fermented liquid was examined for alcohol and volatile acid, but none was found. A small quantity of non-volatile acid was obtained by extracting the residue from the volatile acid determination with ether, but the amount was too small to permit of identification.

The curves given in Fig. 1 show graphically the rate of carbon dioxide production and are drawn from calculations made on the basis of 100 cc. Since portions of the fermenting solution were removed at irregular intervals, it is not possible to construct curves directly from the data given in the tables.

TABLE VI.

Evolution of Carbon Dioxide from Xylose by Penicillium glaucum, No. 6.

Experiment 6.				Experiment 7.			
Age of culture.	CO₂ evolved.		Sugar fermented.	Age of culture.	CO₂ evolved.		Sugar fermented.
	Rate.	Total.			Rate.	Total.	
$hrs.$	$gm.$	$gm.$	$gm.$	$hrs.$	$gm.$	$gm.$	$gm.$
48	0.176	0.176		47	0.150	0.150	
55	0.181	0.357		69	0.402	0.552	
77	0.571	0.928		90	0.713	1.265	
97	0.650	1.578		114	0.588	1.853	
125	0.833	2.411		144	0.537	2.390	
151	0.455	2.866		185	0.493	2.883	3.21
169	0.268	3.134		280	0.209	3.092	
193	0.244	3.378		405	0.074	3.166	
216	0.243	3.621	4.60	671	0.094	3.260	3.21
Weight of pad .... 1.433 gm.				Weight of pad .... 0.730 gm.			

Fermentation by Penicillium glaucum.—Two experiments similar to those with *Aspergillus niger* were carried out with *Penicillium glaucum.* A comparison of the two molds shows that *Penicillium glaucum* produced a smaller weight of pad, but a larger quantity of carbon dioxide. Expressed in figures *Penicillium glaucum* produced about 70 per cent as much growth as *Aspergillus niger*, but 25 per cent more carbon dioxide. Evidently cellular activity is much greater in the case of *Penicillium glaucum.* Slightly higher figures were also obtained for the percentage of total carbon represented by the carbon dioxide. At the time all of the sugar was destroyed (Experiment 7), the figure was 62 per cent and

at the end of the experiment the figure had risen to 70 per cent. The data are given in Table VI.

The fermented liquid was examined for other products such as alcohol and volatile acid, but neither of these was found. During the fermentation a slight acidity developed which seemed to be due to non-volatile acids, but the amount was too small to show any positive tests for either oxalic or citric acid.

A noticeable difference between the two molds was the failure of the gases from *Penicillium glaucum* to discolor sulfuric acid. It apparently does not give off a volatile compound such as is produced by *Aspergillus niger*.

Elementary Composition of Mycelium.

In order to determine the disposition of sugars in the metabolism of molds and other oxidizing organisms, a carbon balance is necessary. An elementary analysis of carbon and hydrogen was made by combustion in an atmosphere of oxygen. The nitrogen was later determined by the Kjeldahl method.

In the combustion of the mold growth, considerable difficulty was at first experienced in getting duplicate determinations. After several trials it was found that by using potassium chromate in the combustion tube and by heating the sample very slowly in a current of oxygen complete oxidation of the material occurred, and good duplicate determinations were secured.

At the end of the combustion the boat was weighed to determine the unburned residue or ash, and the figures thus obtained are included in Table VII.

In general *Penicillium glaucum* contains a higher percentage of carbon, hydrogen, nitrogen, and ash than *Aspergillus niger*. This is particularly true for carbon where the average is 49 per cent for *Penicillium glaucum*, and 46 per cent for *Aspergillus niger*. Smaller differences appear in the figures for hydrogen, nitrogen, and ash, but the difference in all cases is in favor of *Penicillium glaucum*.

Balance of Products.—From the data given in Tables IV, V, VI, and VII for xylose consumed, carbon dioxide produced, and weight and percentage composition of the mycelium, a carbon balance can be calculated. The results are given in Table VIII and show that from 90 to 95 per cent of the carbon of the xylose

is accounted for by the carbon dioxide and mycelium. The remainder may be accounted for by dissolved carbon dioxide, organic acids, and other fermentation products. Mazé in four experiments with *Eurotyopsis gayoni* recovered in the metabolism products from 88 to 97 per cent of the carbon. In the

TABLE VII.

Elementary Composition of Mycelium.

Name.	Age of culture.	Referred from Table.	Carbon.	Hydrogen.	Nitrogen.	Ash.
	days		per cent	per cent	per cent	per cent
A. *niger*, No. 4.	9	IV	46.9	8.00	4.6	
" *sp.*, No. 1.	14	V	47.3	7.76	4.7	4.0
" *niger*, No. 5.	14	V	46.0	6.97	4.2	5.6
" " " 5.	7	VI	44.5	7.18	4.5	6.4
" " " 5.	28	VI	45.4		4.2	
P. *glaucum*, No. 6.	14	V	46.3	7.50	5.0	5.9
" " " 6.	9	VII	50.7	7.44	5.7	7.5
" " " 6.	29	VII	49.7	8.03	5.9	4.5

TABLE VIII.

Balance between Carbon of Xylose and Carbon of Products.

Name.	Age of culture.	Referred from Table.	Carbon contained in				Carbon recovered.
			Xylose.	CO_2	Mycelium.	Total products.	
	days		gm.	gm.	gm.	gm.	per cent
A *sp.*, No. 1.	14	V	0.80	0.493	0.267	0.760	95.0
" *niger*, No. 5.	14	V	0.80	0.468	0.317	0.785	98.0
" " " 5.	7	VI	1.37	0.568	0.636	1.214	88.0
" " " 5.	28	VI	1.28	0.702	0.469	1.271	91.0
P. *glaucum*, No. 6.	14	V	0.59	0.351	0.185	0.536	91.0
" " " 6.	9	VII	1.84	0.989	0.727	1.715	93.0
" " " 6.	28	VII	1.28	0.890	0.363	1.253	98.0

single experiment which he carried out, Waterman obtained a figure of 96 per cent. The close approach to 100 per cent is in harmony with previous evidence indicating the absence of any appreciable quantities of oxalic or citric acids.

A comparison of the 28 day cultures of the two molds shows the same relations as have already been noted, namely more carbon

converted into carbon dioxide, and less into mycelium by *Penicil-lium glaucum* than by *Aspergillus niger*.

Ratios of Products to One Another and to Sugar Consumed.—The relations existing between the products of metabolism and the compound fermented have been expressed by means of various ratios or percentages. Such terms as respiration coefficient, economic coefficient, plastic equivalent, and respiration equivalent, have been introduced into the literature. Some confusion and lack of uniformity in the employment of these terms exist. In order to make perfectly clear the meaning given to these terms in

TABLE IX.

Relations of Metabolism Products to One Another and to Sugar Consumed.

	A. niger, No. 1.	A. niger, No. 5.			P. glaucum, No. 6.		
Age of culture, *days*	14	14	7	28	14	9	28
Respiration coefficient, $\dfrac{\text{wt. of } CO_2}{\text{wt. of dry fungus}}$	3.2	2.5	1.5	2.5	3.2	2.5	4.5
Economic coefficient, $\dfrac{\text{wt. of xylose consumed}}{\text{wt. of dry fungus}}$	3.6	2.9	3.5	2.4	3.7	3.2	4.4
Respiration equivalent, $\dfrac{C \text{ of } CO_2}{C \text{ of xylose}} \cdot 100$	62	59	41	55	60	54	70
Plastic equivalent, $\dfrac{C \text{ of fungus}}{C \text{ of xylose}} \cdot 100$	33	40	47	36	31	39	28

this paper, a formula is placed at the head of each column in Table IX to show how the different values were obtained. The calculations are based on the data of Tables IV, V, VI, and VIII.

The respiration coefficient increases and the economic coefficient decreases with the age of the culture. Both ratios are greater for *Penicillium glaucum* than for *Aspergillus niger* and express by means of a number the more profuse growth of *Aspergillus niger*.

Perhaps the most interesting relations are found in the percentages of carbon converted into carbon dioxide, and into mold growth. As is to be expected, the respiration equivalent increases and the plastic equivalent decreases with the age of the culture. In the early stages of growth, the mold stores a consider-

able quantity of carbon material in the cells, and later converts this material into carbon dioxide and other products. The respiration equivalent is, therefore, inversely proportional to the plastic equivalent.

SUMMARY.

Of the 25 species of molds studied 16 were found to ferment the pentoses with rapidity. Most of the remaining 9 cultures grew slowly although 3 or 4 produced only a few mycelian threads. The best fermenters were found among the *Aspergilli* and *Penicillia* species although a number of molds of these types attacked the pentoses but slowly. The *Mucors*, *Rhizopus nigricans*, and *Cunninghamella*, were also found to be very slow fermenters. From these results it is suggested that pentoses might be of considerable value in the separation and classification of fungi.

4 or 5 days sufficed for the complete destruction of 4 per cent solutions of the sugars with the most active forms of *Aspergillus* and *Penicillium*. No marked difference manifested itself in the rates of fermentation of the two pentoses. With the slower growing molds, xylose seemed to be slightly superior to arabinose. Compared with glucose the two pentoses were fermented somewhat less rapidly.

Carbon dioxide and mycelium represented the major portion of the sugars consumed. Carbon dioxide represented from 40 to 70 per cent and mycelium from 28 to 47 per cent of the carbon of the xylose. In these two products is recovered from 88 to 98 per cent of the total carbon consumed.

The percentage of total carbon represented by carbon dioxide varied with the species and age of the mold. *Penicillium glaucum* produced about 70 per cent as much mycelium as *Aspergillus niger* and about 25 per cent more carbon dioxide. The rate of evolution of carbon dioxide production was also faster with *Penicillium glaucum*. The maximum rate of carbon dioxide production was reached 3 or 4 days after the surface of the liquid became well covered with mycelium.

A small quantity of non-volatile acid was formed, but the amount was insufficient for identification. Tests for oxalic and citric acids were negative. No alcohol or volatile acid could be detected.

Aspergillus niger produced a substance volatile at room temperature, which discolored sulfuric acid. This discoloration may be due to the substance which gives the mold its characteristic odor. *Penicillium glaucum* did not produce any such substance.

An analysis of the dry fungus showed that *Penicillium glaucum* contained more carbon, hydrogen, nitrogen, and ash than *Aspergillus niger*. The most marked difference was found in the carbon content; 49 per cent for *Penicillium glaucum* and 46 per cent for *Aspergillus niger*. About 5 per cent of nitrogen in the dry fungus indicates the presence of a considerable quantity of organic nitrogenous material in the mold growth. The ash content was also found to be about 5 per cent.

BIBLIOGRAPHY.

1. Lafar, F., Handbuch der Technischen Mykologie, Jena, 1905–07, iv, 236, 268.
2. Steinberg, R. A., *Am. J. Bot.*, 1919, vi, 330.
3. Behrens, J., *Centr. Bakt., 2te Abt.*, 1898, iv, 547.
4. Went, F. A. F. C., *Centr. Bakt., 2te Abt.*, 1901, vii, 591.
5. Emmerling, O., *Centr. Bakt., 2te Abt.*, 1903, x, 273.
6. Krüger, F., *Arb. k. Biol. Anst. Landw. Forst.*, 1913, ix, 233.
7. Hawkins, L. A., *Am. J. Bot.*, 1915, ii, 375.
8. Weimer, J. L., and Harter, L. L., *J. Agric. Research*, 1921, xxi, 189.
9. Waterman, H. I., *Folia microbiol.*, 1912, i, 422; 1913, ii, 135.
10. Mazé, P., *Compt. rend. Acad.*, 1902, cxxxiv, 191, 240. Kosinski, I., *Jahrb. wissensch. Bot.*, 1902, xxxvii, 137. Watterson, A., *Bull. Torrey Bot. Club*, 1904, xxxi, 291. Buromsky, J., *Centr. Bakt., 2te Abt.*, 1912–13, xxxvi, 54. Stoklasa, J., *Centr. Bakt., 2te Abt.*, 1911, xxix, 401. Harter, L. L., and Weimer, J. L., *J. Agric. Research*, 1921, xxi, 211.
11. Fred, E. B., Peterson, W. H., and Davenport, A., *J. Biol. Chem.*, 1919, xxxix, 347. Fred, E. B., Peterson, W. H., and Anderson, J. A., *J. Biol. Chem.*, 1921, xlviii, 385.
12. Scales, F. M., *Science*, 1920, li, 214.
13. Van Slyke, D. D., *J. Biol. Chem.*, 1917, xxx, 347.

THE INFLUENCE OF THE SODIUM ION IN THE PRODUCTION OF TETANY.*

By FREDERICK F. TISDALL.

(From the Nutritional Research Laboratories of the Hospital for Sick Children and the Department of Pediatrics, University of Toronto, Toronto, Canada.)

Binger (1) in 1917 reported the production of tetany in dogs by the intravenous injection of o-phosphates. He concluded as follows.

"When the phosphate solution is injected in amounts equivalent to 150 mgm. phosphorus per kilogram (of body weight) the serum calcium drops from its normal level of 10 mgm. per 100 cc. to approximately 6 mgm.

"At this level a condition of tetany supervenes, provided the neutral or alkaline salts have been injected. With acid phosphate solutions, the calcium drop occurs unaccompanied by tetany."

Jeppsson and Klercher (2) produced electrical excitability in both animals and children by feeding large amounts of alkaline phosphates and Howland and Marriott (3) and others have reported the production of tetany by the oral or intravenous administration of sodium bicarbonate. In a study of the inorganic constituents in the serum of infants with tetany it was found by Kramer, Tisdall, and Howland (4) that the concentration of sodium in the serum was essentially unaltered. These authors found a diminished calcium content of the serum, which they considered was the cause of the increased irritability of the neuromuscular mechanism. Collip and Backus (5) and later Grant and Goldman (6) and others produced tetany in the adult by prolonged voluntary hyperpnea. No diminution in the calcium content of the serum was found by the latter investigators.

35

The present investigation was undertaken in an endeavor to determine the relation of some of the different inorganic constituents of the serum to the production of tetany. Dibasic sodium phosphate (J. T. Baker) and phosphoric acid were injected intravenously into dogs, the time required for each injection being about 1 hour. The effect of the procedure on the sodium, potassium, calcium, inorganic phosphorus, and chlorine content, and the pH and CO_2-combining power of the serum were then determined. The blood was removed from the femoral vein before the injection was begun, and 1 hour after its completion. It was collected, under oil, the serum separated and the CO_2 and pH determined as soon as possible. The CO_2 in the serum was determined directly by means of the Van Slyke apparatus, without bringing it into equilibrium with the alveolar air. The pH was determined colorimetrically by a method devised by Cullen (7), the chlorine by a combination of the Van Slyke and Donleavy (8) and Volhard methods, the sodium, potassium, and calcium by methods devised by Kramer and Tisdall (9, 10, 11) and the inorganic phosphorus by a method devised by the author (12).

The dibasic sodium phosphate was injected in Dogs 1 to 4 (Table I) while the phosphoric acid was given to Dogs 5, 6, and 7. The phosphoric acid produced no apparent toxic effects. The respirations did not increase above 30 per minute and in all the dogs the pulse remained excellent throughout. After the completion of the injections the dogs jumped up and ran about in a normal manner. No later ill effects were observed. On the other hand the dibasic sodium phosphate produced signs of profound toxemia. The respirations were increased to 30 to 100 per minute and the pulse was increased to 125 to 180 per minute. After the injection the dogs had great difficulty in standing, and vomited at frequent intervals. In all four dogs some twitching of the facial muscles and also spasm of the jaw muscles occurred, but in only Dog 4 did marked twitching of the whole body (tetany) occur. The dogs were carefully observed for 6 to 7 hours after the administration of the phosphates but no essential changes occurred in their condition. On the morning following the experiments, Dogs 1, 3, and 4 were found dead while Dog 2 although still showing signs of toxemia was found to be alive. This animal recovered perfectly.

It is seen that the injection of the dibasic sodium phosphate caused some twitching in all four dogs although a condition resembling active tetany occurred in only one dog. After the in-

TABLE I.

Intravenous Injections of Dibasic Sodium Phosphate and Phosphoric Acid.

Dog.	Twitching.	Solution injected	Amount of solution injected (cc.)	Amount of P per kilo. (mg.)	Weight of dog. (kg.)	Blood obtained before and after injection.	Ca per 100 cc. of serum. (mg.)	Inorganic P per 100 cc. of serum. (mg.)	Na per 100 cc. of serum. (mg.)	K per 100 cc. of serum. (mg.)	Cl per 100 cc. of serum. (mg.)	CO2-combining power. (vol. per cent)	pH
1	Slight.	Na_2HPO_4	480	144	14.6	Before.	10.1	6.1	336	19.2	388	50	7.40
						After.	5.6	13.3	335	19.3	388	49	7.35
2	"	Na_2HPO_4	280	150	9.8	Before.	10.2		338	19.4	381	56	7.45
						After.	7.0	15.5	346	20.2	381	84	7.45
3	"	Na_2HPO_4	300	170	7.5	Before.	10.6	6.0	348	21.3		60	
						After.	6.4	32.0	350			56	7.45
4	Marked.	Na_2HPO_4	425	150	11.4	Before.	10.9	3.0		22.5	388	58	7.40
						After.	5.7	17.0	353	20.0	380	58	7.50
5	Absent.	H_3PO_4	250	150	4.6	Before.	9.8	5.3	335	20.3	388	58	7.40
						After.	5.5	13.4	298	16.0	367	34	7.25
6	"	H_3PO_4	450	150	16.0	Before.	10.5	2.9	347	20.1	381	66	
						After.	6.5	22.5	320	20.2	381	44	7.40
7	"	H_3PO_4	400	180	10.5	Before.	10.9	6.8	334	18.8	381	58	7.35
						After.	6.3	19.6	305	18.4	381	36	7.20

jection of the phosphoric acid no signs of increased irritability appeared. It is not unreasonable to consider the slight muscular twitchings and spasm of the jaw muscles in the first group of animals as signs of incipient tetany.[1]

[1] Binger (1) apparently obtained signs of active tetany in every instance when he injected the dibasic phosphate in the amounts used in the present study. The difference in the results obtained might be due to the osmotic

The following changes in the blood serum were found to occur after the intravenous injection of dibasic sodium phosphate (Table I): (a) A reduction of the calcium from an average of 10.5 mg. per 100 cc. to 6.2 mg.; (b) an increase of the inorganic phosphorus from about 5.0 mg. per 100 cc. to an average of 19.0 mg.; and (c) a change of the pH from 7.40 to 7.50 in one dog only.

No definite increase occurred in the sodium content of the serum. No essential change occurred in the concentration of potassium and chlorine. No marked change occurred in the CO_2 content except in Dog 2, in which it was increased from 56 to 84 volumes per cent.

The following changes occurred in the blood serum after the intravenous injection of phosphoric acid: (a) A reduction of the calcium from an average of 10.4 mg. per 100 cc. to 6.1 mg.; (b) an increase of the inorganic phosphorus from about 5.0 mg. per 100 cc. to an average of 18.5 mg.; (c) a decided reduction of the sodium and the CO_2-combining power of the serum; and (d) a definite increase in the acidity of the serum in two of the three dogs.

No essential change occurred in the potassium and chlorine.

Reference has already been made to the production of tetany in normal adults by prolonged voluntary hyperpnea (5, 6). Collip and Backus, and Grant and Goldman independently advanced the following theory to explain this phenomenon. The excessive breathing "washes out" the carbon dioxide from the blood. This tends to alter the normal ratio $\frac{H_2CO_3}{NaHCO_3}$ (which is one of the important factors in the neutrality mechanism (13)), by decreasing the numerator. To maintain this normal ratio there must also be a decrease in the denominator. This necessi-

pressures of the phosphate solutions. The solutions used by Binger were about m/15 and thus contained about 200 mg. of P per 100 cc. The solution injected into Dogs 1 to 4 in the present experiment contained respectively 438, 514, 430, and 430 mg. of P per 100 cc., which makes them considerably more hypertonic than those used by Binger.

tates a rapid excretion of sodium bicarbonate from the blood, and if this is not excreted rapidly enough by the kidneys, the excess according to this theory is absorbed by the tissues. Thus there is an increase in the tissues of the irritating sodium salt and tetany results.

If we apply this theory to the present work in which the dibasic sodium phosphate was injected we would then consider that the excess of sodium phosphate which could not be excreted in such a short time was absorbed by the tissues. The calcium in the tissues was probably diminished in an attempt to come in equilibrium with the lower content in the serum which was produced by the injected phosphorus. Thus the increased sodium combined with a lowered calcium resulted in the production of tetany.

Not the slightest signs of tetany were produced in Dogs 5, 6, and 7, by the injection of phosphoric acid. In these animals the calcium was reduced and the inorganic phosphorus increased to about the identical levels present in the first four dogs, which received the sodium phosphate. However, the sodium in the serum, and no doubt in the tissues, was lowered in the process of neutralization and excretion of the injected acid. This lowered sodium apparently explains the absence of tetany after the injection of phosphoric acids.

It has been found by Loeb (14), Mathews (15), and others that the anions are not without an effect on the maintenance of a normal irritability of the muscle and nerve cells. Loeb found that when the minimal stimulating concentration of sodium chloride was 0.0625 M, the same effect was produced by an 0.0625 to 0.03125 M solution of sodium bicarbonate and a M/128 solution of dibasic sodium phosphate. In the present experiment there is no evidence that the phosphates have any definite effect in the production of tetany such as the work of Loeb would indicate.

In some of the dogs a definite change occurred in the pH of the serum. Attention has been drawn to the fact that calcium salts tend to become less ionized in alkaline solutions (16). The converse is true. It is highly improbable, however, that the presence or absence of tetany was due to a decreased or increased ionization of the calcium salts.

The theory advanced by Collip and Backus and Grant and Goldman offers an adequate explanation for the development of tetany after the administration of sodium bicarbonate reported by Howland and Marriott (3) and Harrop (17). In the cases (infants) reported by Howland and Marriott there is considerable evidence that the kidney function was not normal (diagnoses, burn, severe diarrhea, and pyelitis). Harrop's patient was an adult with anuria. Applying this theory, we may assume that tetany may be produced by a retention of sodium salts due to either a defective action of the kidneys or the injection of large quantities of sodium under such conditions that the normal kidney action cannot remove it rapidly enough from the body to prevent an accumulation in the tissues.

Gastric Tetany.

It is of interest to note the blood changes which occur in gastric tetany. MacCallum and his coworkers (18) produced tetany in dogs by obstruction of the pylorus. These investigators found that with the production of tetany there was an increase in the CO_2-combining power of the plasma and a decrease in the chlorides. In somewhat similar experiments Hastings, Murray, and Murray (19) also found an increase in the CO_2-combining power of the plasma and a decrease in the chlorides. In addition the sodium in the serum was found to be decreased (in two out of three dogs) and the calcium slightly increased. During the past 2 years, the author has had the opportunity of estimating the calcium in the serum in two patients with gastric tetany. In both cases the calcium content was normal: 10.0 and 10.6 mg. per 100 cc. In one case[2] the sodium, potassium, chlorine, and CO_2-combining power of the serum were also determined. The concentrations found in this case and the average normal values per 100 cc. of serum are as follows: Na 287 mg., normal 335 mg.; K 10.6 mg., normal 20.0 mg.; Cl 215 mg., normal 360 mg.; CO_2-combining power 103 volumes per cent, normal 65 volumes per cent.

[2] This was an adult patient in the Johns Hopkins Hospital, Baltimore. The author is indebted to Dr. W. W. Palmer for the chlorine and CO_2 values which were determined in his laboratory. The calcium and potassium concentrations were determined by the author in the laboratory of the Department of Pediatrics, Johns Hopkins University.

It is seen that the sodium-calcium ratio is disturbed in the opposite direction to that which occurs in other types of tetany. Probably the most significant change is the marked increase in the bicarbonate anion. The importance of this anion has been emphasized by Collip (16) who considers it may have a specific action in the production of tetany.

It is thus evident that tetany may develop notwithstanding a diminished ratio of sodium to calcium in the serum.

CONCLUSIONS.

1. The sodium-calcium ratio is the important factor in the production of tetany, with the exception of the gastric type.

2. Gastric tetany is not due to a disturbance of the sodium-calcium ratio but apparently to an increase of the bicarbonate ion.

3. No evidence has been found that the calcium-phosphorus ratio has any influence on the production of tetany.

BIBLIOGRAPHY.

1. Binger, C., *J. Pharmacol. and Exp. Therap.*, 1917–18, x, 105.
2. Jeppsson, K., and Klercher, O., *Z. Kinderh.*, 1921, xxviii, 71.
3. Howland, J., and Marriott, W. McK., *Quart. J. Med.*, 1917–18, xi, 289.
4. Kramer, B., Tisdall, F. F., and Howland, J., *Am. J. Dis. Child.*, 1921, xxii, 431.
5. Collip, J. B., and Backus, P. L., *Am. J. Physiol.*, 1920, li, 568.
6. Grant, S. B., and Goldman, A., *Am. J. Physiol.*, 1920, lii, 209.
7. Cullen, G. E., *J. Biol. Chem.*, 1922, l, p. xvii.
8. Van Slyke, D. D., and Donleavy, J. J., *J. Biol. Chem.*, 1919, xxxvii, 551.
9. Kramer, B., and Tisdall, F. F., *J. Biol. Chem.*, 1921, xlvi, 467.
10. Kramer, B., and Tisdall, F. F., *J. Biol. Chem.*, 1921, xlvi, 339.
11. Kramer, B., and Tisdall, F. F., *J. Biol. Chem.*, 1921, xlvii, 475.
12. Tisdall, F. F., *J. Biol. Chem.*, 1922, l, 329.
13. Henderson, L. J., *Am. J. Physiol.*, 1908, xxi, 173.
14. Loeb, J., *Arch. ges. Physiol.*, 1902, xci, 248.
15. Mathews, A. P., *Am. J. Physiol.*, 1904, xi, 455.
16. Collip, J. B., *Am. J. Physiol.*, 1920, lii, 483.
17. Harrop, G. A., Jr., *Bull. Johns Hopkins Hosp.*, 1919, xxx, 62.
18. MacCallum, W. G., Lintz, J., Vermilye, H. N., Leggett, T. H., and Boas, E., *Bull. Johns Hopkins Hosp.*, 1920, xxxi, 1.
19. Hastings, A. B., Murray, C. D., and Murray, H. A., Jr., *J. Biol. Chem.*, 1921, xlvi, 223.

STUDIES OF HUMAN MIXED SALIVA.

I. THE DETERMINATION OF THE HYDROGEN ION CONCENTRATION OF HUMAN MIXED SALIVA.*

By HENRY E. STARR.

(From the Robert Hare Laboratory of Chemistry, School of Medicine, University of Pennsylvania, Philadelphia.)

INTRODUCTION.

Variations in the composition of human mixed saliva, *i.e.* saliva as it normally is found in the mouth, consisting of a mixture of the secretions of the submaxillary, sublingual, parotid, and buccal glands, have been noted in various conditions of health and disease by countless investigators. The salivary reaction, particularly, has been the subject of many reports for almost a century.

In 1835, Donné (1) reported that although he found the saliva normally alkaline to litmus, he had found it acid in many diseases, including encephalitis. In 1844, Wright (2) attempted to elaborate a diagnostic system of salivary analysis. About the same time, Simon (3) wrote at length on "morbid saliva" as distinct from "normal." Among other studies of a similar character were those of Binet (4) in 1884, Sticker (5) in 1889, Dieminger (6) in 1898, Michaels (7) in 1902, and Fleckseder (8) in 1916. Since 1901, Kirk has been the chief exponent of the concept of "the saliva as an

---

index of faulty metabolism" (primarily in relation to the etiology of dental caries) and has conducted and fostered many investigations (9–14). In 1916, Marshall (15–19) studied the salivary reaction in various physiological and pathological conditions, including dementia præcox and epilepsy, by means of a titration technique ("salivary factor" and "total neutralizing power") which he had developed for the study of dental caries. In 1918, Ludlum (20) reported two sets of litmus paper reactions of successive specimens of saliva derived from two subjects whom he regarded as representing different types of insanity. The chemistry of the saliva, especially as a factor in dental hygiene, and the quantitatively determined salivary reaction in relation to the nature of substances taken into the oral cavity, has been intensively studied by Gies (21–28), Pickerill (29), and Prinz (30–32). Since 1919 Sullivan and his colleagues (33, 34) have been investigating the saliva of pellagra patients at various stages of the disease, employing refined quantitative methods, with especial reference to sulfocyanate content.

The peculiarly contradictory findings of many investigators, the innervation of the salivary glands by both cranial and sympathetic nerves, and the variation in quality and quantity of secretion depending upon the nerve stimulated (see Heidenhain (35) and Langley (36)); the adaptation of secretion to the ingested substance and the reflex secretion arising from purely psychic stimuli (Pawlow, 37); together with the dry mouth of fear (considered by Cannon, 38) and the drooling of the idiot:—these factors render the problem of correlation between salivary composition and metabolic disorder a promising field for research.

Almost all of the investigators mentioned were interested in the salivary reaction as the principal variant. The early investigators employed the crude litmus paper test, reporting specimens simply as "acid" or "alkaline." Probably the first reliable quantitative determination of the degree of acidity or alkalinity of human mixed saliva was that of Chittenden and Ely (39) who, in 1883, found the average alkalinity for fifty-one specimens as determined by titration with cochineal as indicator, to be 0.08 per cent "expressed as sodium carbonate."

A subsequent series by Chittenden and Smith (40) published in 1885 gave a mean of 0.097 per cent similarly expressed. Schlesinger (41) obtained somewhat lower findings, reporting, in 1891, an average of 0.032 per cent, also expressed as sodium carbonate. Other investigators have reported on the alkalinity of the saliva expressed in terms of sodium hydroxide, among which may be mentioned Szabo (42) who, in 1900, found the

alkalinity of the saliva to vary between 0.058 and 0.064 per cent. Cohn (43) also in 1900, reported the alkalinity as varying from 0.002 to 0.048 per cent expressed as NaOH. Probably the most extensive series of determinations of both "acidity" and "alkalinity" of human mixed saliva was that conducted by Gies (26) in 1916. In his report, Gies makes the statement that his results "have shown that salivary secretion varies, both qualitatively and quantitatively, within limits that are independent of any mechanical stimulus or any dental condition"—a highly suggestive observation from the point of view of the present investigation.

In each of the above mentioned researches, all quantitative determinations were made by titration, the indicators most frequently employed being litmus, cochineal, lacmoid, methyl orange, p-nitrophenol, and phenolphthalein. The work was, consequently, on the "quantity factor" rather than the "intensity factor" of the acidity.

Very few investigations of the actual hydrogen ion concentration of saliva have been reported. Foa (44) is usually cited in this connection, and his findings, by the electrometric method, were reported in 1906. He found the mixed saliva before and after eating to have a hydrion concentration corresponding respectively to pH 8.2 and 8.3.

Kirk (12) published a series of determinations made by means of the Hildebrand hydrogen electrode in 1914. His findings were pH 7.9, 5.6, 6.4, 7.7, 8.1, 7.5, 6.4, 6.2, and 8.6.

Michaelis and Pechstein (45) in the course of an investigation of conditions affecting the activity of salivary diastase, in 1914, reported electrometric determinations of the hydrion concentration of three samples of saliva, undiluted; two samples diluted with "gewöhnlichem" distilled water (presumably containing some CO_2); and one specimen diluted with recently boiled freshly distilled water (i.e., approximately carbon dioxide-free). In each instance of dilution 1 volume of saliva was diluted with 9 volumes of water. Their results were: undiluted saliva, pH 6.79, 6.91, and 6.92; saliva diluted with "gewöhnlichem" distilled water, pH 6.65 and 6.34; saliva diluted with freshly boiled recently distilled water, pH 7.01. Their conclusion (Michaelis and Pechstein (45), p. 92) is "Da die neutrale Reaktion bei 7.07 (18°) liegt, so ist also der Speichel leicht sauer."

A careful study of the literature has revealed only the above cited researches of Foa, Kirk, and Michaelis and Pechstein as stating definitely the hydrion concentration of human saliva, electrometrically determined. As to colorimetric determinations, Graham (46) made some pH determinations in an investigation of litmus as an indicator of the salivary reaction within certain limits. In 1920, Bloomfield and Huck (47) in the course of an investigation of the bacteria of the oral cavity, colorimetrically determined the pH of a number of specimens of saliva. Unfortunately in the former investigation the saliva was dialyzed and

in the latter it was centrifuged, both procedures no doubt resulting in loss of CO_2 and resultant increase in pH, as indicated in a subsequent section. It is of interest to note in this connection that in 1916 Gies pointed out that centrifugation of mixed saliva results in increase of the supernatant liquid's alkalinity to phenolphthalein (25).

The suggestive fluctuations of the "quantity factor" of acidity of mixed saliva as reported by many investigators, the meagerness of data upon the "intensity factor" or hydrion concentration, especially as regards colorimetric determinations thereof, together with a realization of the ease with which such determinations might be made by adapting the technique instituted by Sørensen (48), Henderson and Palmer (49), Clark (50), and Cullen (51, 52) led directly to the experiments reported in this article.

<div align="center">EXPERIMENTAL.</div>

<div align="center">*Methods.*</div>

The method which has been found most satisfactory and which has been employed throughout the present investigation (except when otherwise stated in the text) is briefly as follows.

The saliva is collected under oil[1] in a clean receiver. 1 cc. is then removed by means of a pipette and transferred to a test-tube containing 9 cc. of freshly boiled distilled water (pH 6.6 to 6.7) and 1 cc. of 0.01 per cent aqueous solution of dibromothymolsulfonephthalein also under oil. The saliva and diluent are then mixed thoroughly by stirring with the flattened end of a glass rod beneath the level of the supernatant oil layer.[1] After the diluted saliva is of uniform virage throughout,[2] the pH is determined by comparison with suitable standards against a milk-glass background.

<div align="center">*Notes.*</div>

1. Preliminary to using any article coming into contact with the saliva —test-tube, pipette, etc.—it is washed and rinsed thoroughly with distilled water and then tested with a few cc. of dilute indicator solution (*e.g.*, 0.001 per cent brom-thymol blue).

---

[1] As in Cullen's method for determination of plasma pH (see Cullen, G. E., *J. Biol. Chem.*, 1922, lii, 501).

[2] The term "virage" was suggested by Sørensen and adopted by Clark (Clark, W. M., The determination of hydrogen ions, Baltimore, 1920, 38).

2. When distilled water was boiled vigorously for a few minutes and then allowed to cool to about 18°C. in a tightly closed glass-stoppered flask, the pH was approximately pH 6.6 to 6.7. Further boiling may result in a higher pH. However, pH 6.6 to 6.7 has been chosen as standard for the water employed in this investigation, as it is more readily attained and maintained than is a higher pH value.

3. Brom-thymol blue as employed serves very well for the determination of hydrion concentrations between pH 5.2 and 7.2, thus including the normal range of salivary pH. For values above pH 7.00 phenol red (0.005 per cent) has been employed to advantage in the same manner. The actual quantity of indicator added is such as to give quite distinguishable virages over the range above indicated. The percentage of the solution employed is such that 1 cc. may be added instead of the usual drop or drops. 1 cc. may be measured more accurately by means of a pipette than can a drop by means of a rod or dropper. Moreover, the solution is thereby rendered so dilute that a slight error in the measurement thereof will have but a negligible effect upon the total amount of indicator added, thus tending to keep more nearly standard the conditions of intensity and brilliance of virage.

In lieu of preparing for each test a mixture of 1 cc. of indicator and 9 cc. of distilled water (pH 6.6 to 6.7), 10 cc. of a stock indicator solution of one-tenth the strength of that cited may be employed, in which case the pH of the diluent is obvious without further testing.

4. Sørensen's standards of M/15 primary potassium phosphate and secondary sodium phosphate solutions, prepared from Merck's chemicals were employed. They covered the range from pH 5.0 to 8.0 at intervals of 0.1 pH. Readings were made to 0.05 pH. Each standard is prepared in a manner similar to that employed for the salivary pH determinations and is kept sealed in hard glass ampules for more or less "permanent" use. It is necessary to check up the series from time to time by comparison with freshly prepared standards—a precaution employed weekly during the present investigation.

The use of the Walpole comparator, or the method of superposition, is seldom necessary as the saliva is but rarely colored. It is, however, frequently quite turbid and it is because of this interfering turbidity that dilution and the milk-glass backing are employed.

Dilution.—The saliva is diluted in the ratio of 1 : 9 with distilled water, which is practically the same dilution as employed by Michaelis and Pechstein (45) and also by Bloomfield and Huck (47). To determine the effect of such dilution upon the salivary pH, a quantity of saliva was collected under oil and portions of it were transferred by means of a pipette to a series of test-tubes containing (also under oil) such amounts of distilled water as to render the proportion of saliva to water successively: 1 part of

saliva to 0, 1, 3, 5, 7, 9, 12, 15, and 20 parts of water. In each tube there had been previously placed and mixed with the water such a quantity of 0.01 per cent brom-thymol blue solution that the indicator was always present in the proportion of 1:10 with respect to the total quantity of *diluted* saliva. Fifty specimens of saliva were thus investigated. The results are indicated in Fig. 1. The initial pH of the salivas examined ranged from 5.90 to 6.85. Temperature was at all times between 18 and 20°C.

Fig. 1. Dilution curve of human mixed saliva. Increases in pH given in 0.01 pH terms, are average values. The mean deviation from the average increase at plateau level was less than 0.05 pH.

It is evident that the saliva undergoes a slight increase in pH on dilution up to a proportion of 1:3 or 1:5, when a plateau is reached and maintained at least until the dilution is 1:12. The slight increase noted (in 94 per cent being 0.05 pH or less) is of relatively little importance in this investigation, inasmuch as readings were made only to 0.05 pH.

It should be noted, however, that dilution of the saliva in the ratio of 1:5 was accompanied by an increase in 30 per cent of the specimens of 0.05 pH and 6 per cent increased 0.10 pH. The values of pH found by this method of dilution should, therefore, probably be reduced by 0.05 pH to obtain a value more closely approximating that of the undiluted saliva. As to the actual hydrion concentration corresponding to the values found

by the colorimetric method, no definite formula can be given, inasmuch as electrometric determinations to correlate the methods (involving determination of protein error) have not yet been made. In the light of Cullen's work on the colorimetric determination of the pH of blood plasma (52), it is probable that the method herein described yields somewhat too high pH values. In the present investigation, however, this is of relatively slight import inasmuch as the determinations were made for comparison of pH value found for different specimens of saliva when the same method was employed.

Collection of Specimen. Influence of Paraffin as Activator.

The work of Gies, Marshall, and others indicates that "activated" saliva is quantitatively more alkaline than "normal resting saliva." Although it appeared most probable that the same conditions would be found with regard to the salivary pH, the assistance which paraffin might give in facilitating the collection of specimens in quantity rendered it advisable to make a definite trial of its usefulness.

Each subject was seated comfortably before a rack containing three test-tubes, each containing about 1 cc. of oil and a thistle tube leading below the surface of the oil. The saliva was allowed to accumulate in the mouth during 5 minute periods, during which time the mouth was kept closed. The first sample was taken 5 minutes after the subject had seated himself and allowed the saliva to collect normally prior to ejecting it through the funnel tube into the test-tube below the surface of the oil. After ejection of the first sample, the subject was at once given a paraffin cube, previously tested for neutrality, which he chewed fairly vigorously for a second 5 minute period. He then ejected the accumulated saliva, removed the paraffin and "rested" 10 minutes, when he again allowed the saliva to accumulate normally, ejecting it at the expiration of the third 5 minute period. The salivary pH was always determined immediately after ejection. The results thus obtained in a series of ten experiments on five subjects appear in Table I.

The data in Table I show clearly that the use of even so inert an activator as paraffin is precluded in obtaining specimens of saliva, inasmuch as in every instance its use resulted in an increase in pH of from 0.10 to 0.60 pH. In each instance, it may also be noted, the pH decreased to approximately its initial value after removal of the paraffin and 10 minute rest.

TABLE I.

The Effect of Chewing upon the Salivary pH.

| Subject. | Reaction of human mixed saliva collected in 5 min. periods. | | |
	Before chewing.	While chewing.	10 min. after chewing.
	pH	*pH*	*pH*
IFT	6.40	6.95	6.55
	6.65	6.85	6.60
KH	6.25	6.85	6.30
	6.35	6.70	6.30
KN	6.40	6.75	6.55
IG	6.40	6.70	6.35
	6.85	7.05	6.75
BMT	6.75	6.95	6.80
	6.85	7.00	6.80
	6.90	7.00	6.85

Treatment of Specimen after Ejection.

Experiments were made to determine how long after ejection from the mouth human mixed saliva may be exposed to air at ordinary temperatures without undergoing change in hydrion concentration. It appeared probable that the salivary pH would increase more or less rapidly from loss of CO_2, and that this loss would be prevented by covering the saliva with oil, a precaution found necessary by Bloomfield and Huck (47).

Each subject was instructed to retain the saliva as it collected naturally in his mouth until he had a "mouthful" and then to eject it into a small thistle tube leading below the surface of a few cc. of oil in a clean test-tube, in the usual manner. The quantity varied with different individuals, some apparently regarding 1 cc. as a "mouthful," while others furnished as much as 10 cc. at a single ejection. . With care, about 5 cc. could usually be collected. The pH of the saliva was determined immediately after ejection. Approximately half of the saliva was then drawn by means of a pipette from below the surface of the oil and placed in a similar test-tube exposed to the air. At intervals the pH of the saliva in each tube was determined until the supply of the particular specimen, exclusive of dregs, was exhausted.

Twelve specimens were thus examined. Only two of those which were under oil showed any increase after standing for 20

minutes, and they increased by but 0.05 pH. Of the portion exposed to the air, all had increased after 20 minutes exposure from 0.05 to 0.40 pH, and three-fourths of them increased 0.05 to 0.10 pH after but 2 minutes exposure.

It is obvious, therefore, that mixed saliva speedily increases in pH when allowed to stand exposed to air at ordinary room temperatures, and that oil employed as indicated in the preceding description of the general method is a necessary and adequate protection.

Determinations without Oil. Method B.—In certain investigations in the earlier stages of the present research oil was not employed and the saliva was mixed with diluent and indicator by pouring rapidly from one test-tube into another for a period of less than 1 minute. This resulted uniformly in loss of CO_2 and increase in pH, as shown by the results of a series of 100 parallel determinations made in this manner and by the method employing oil as described in the text. The average increase was 0.15 pH, and the average deviation therefrom was less than 0.05 pH. Since the dilution effect is approximately 0.05, the findings of the non-oil method, as employed, may be regarded as uniformly 0.20 pH higher than the pH of the original undiluted saliva. Findings obtained by this method will be reported in certain instances in this series, but will always be accompanied by the statement that they were obtained by the non-oil method.

It should be noted that the non-oil method is not recommended, for lack of care in mixing, or undue prolongation of the pouring can lead to an error of 0.45 pH, or more. 0.15 pH is presented as the increase arising from the method *as employed in this investigation*.

Centrifugation.—Since simple exposure to air results in loss of CO_2 and consequent increase in pH, it would seem evident that centrifugation, in the usual manner,[3] results in even greater loss of CO_2. In 1916, Gies (26) reported that centrifugation of saliva results in decreased acidity of the supernatant layers to phenolphthalein and increased acidity at the lower levels. The following determinations were carried out to determine the effect of centrifugation on mixed saliva, in terms of pH.

20 cc. of mixed saliva were collected from each subject, who was allowed to eject several "mouthfuls" to obtain that quantity. The saliva was collected below a 2 cc. layer of oil in a graduated 25 cc. cylinder in which had

---

[3] For adequate measures to be employed in the centrifugation of carbon dioxide-containing solutions to avoid loss of carbon dioxide, see Cullen, G. E., *J. Biol. Chem.*, 1922, lii, 508.

been placed 2 cc. of the brom-thymol blue solution, the ejections being made into a funnel tube leading below the level of the oil. When the desired amount of saliva had been collected it was thoroughly mixed with the indicator by stirring in the usual manner. 11 cc. were then transferred by means of a pipette to a 15 cc. centrifuge tube containing 1 cc. of oil, below which the saliva and indicator mixture were introduced. The tube was then stoppered tightly with a rubber cork leaving an air-space of about 2 cc. A second 11 cc. portion was placed in a similar centrifuge tube without a protective oil layer and uncorked. The pH was determined by comparison with standards in the same type of centrifuge tubes. Both tubes containing saliva were then centrifuged for 10 minutes at the rate of 2,500 revolutions per minute, when they were removed, the pH of each was again determined and any difference in pH at different levels in the tubes carefully noted. The sediment was then stirred thoroughly with the supernatant liquid in each tube and the pH again determined.

This procedure was repeated on ten specimens obtained from four subjects. The initial pH values of the specimens ranged from pH 6.55 to 6.85. After centrifugation, all the specimens showed an increase in pH of the supernatant liquid. In the open tubes, the increment was from 0.15 to 0.55 pH and averaged 0.30. In the stoppered tubes the increment was from 0.05 to 0.20, averaging less than 0.15 pH. No definite pH value can be given for the lowest stratum of semisolid material which displayed a virage considerably below the limits of accuracy of the indicator employed; i.e., below pH 5.2. After mixing this material, which was markedly present in all but two specimens, with the supernatant liquid, the resultant mixture, in the open tubes, invariably showed a lower pH than did the unmixed supernatant liquid, but not as low as the initial salivary pH. Thus, after mixing, in the open tubes the pH was still 0.10 to 0.30 pH higher than the initial value. In the corked tubes, the mixture was 0.05 to 0.10 higher in pH value than was the initial value.

It is evident, therefore, that centrifugation as employed results in loss of CO_2 and precipitation of a substance of greater hydrion concentration than the supernatant liquid, both of which effects result in increased pH of the supernatant liquid. This increase is greater in the case of saliva exposed to the air than in that partially protected by oil and stopper. In the latter the CO_2 no doubt came into partial equilibrium with the CO_2 in the oil and air-space above the liquid, with consequently less loss of CO_2.

If centrifugation is to be employed at all, it is obvious that rigid precautions must be taken to avoid loss of carbon dioxide. Moreover, precipitation of the acidic sediment (termed "acid mucinate" by Gies (24)) may lead to erroneous findings as to pH value.

CONCLUSION.

A method for the colorimetric determination of the hydrogen ion concentration of human mixed saliva is described, together with the precautions necessary. Activators, such as paraffin, result in increase of salivary pH.

BIBLIOGRAPHY.

1. Donné, A., *Arch. gen. med.*, 1835, viii, series 2, 53, 147.
2. Wright, Der Speichel in physiologischer, diagnostischer und therapeutischer Beziehung, Vienna, 1844.
3. Simon, J. F., Physiologische und Pathologische Anthropochemie mit Berücksichtigung der eigentlichen Zoochemie, Berlin, 1842.
4. Binet, P., Sur la sueur et la saliva dans leur rapport avec l'élimination, Paris, 1884.
5. Sticker, G., *Deutsch. med. Ztg.*, 1889, suppl., No. 18.
6. Diemiuger, H., Beiträge zur Kenntniss des menschlichen Mundspeichels in gesunden und pathologischen Varhaltnissen, Dissertation, Würzburg, 1898.
7. Michaels, J. P., Sialo semeiology, Philadelphia, 1902.
8. Fleckseder, R., *Z. Heilk. Abt. inn. Med.*, 1916, xxvii, 231.
9. Kirk, E. C., *Dent. Rev.*, 1903, xvii, 383.
10. Kirk, E. C., *Dent. Cosmos*, 1910, lii, 729.
11. Kirk, E. C., *Dent. Summary*, 1914, xxxvi, 35.
12. Kirk, E. C., *Dent. Cosmos*, 1914, lvi, 1.
13. Kirk, E. C., *Dent. Cosmos*, 1915, lvii, 33.
14. Kirk, E. C., Crowell, W. S., and Appleton, J. L., Jr., *J. Allied Dent. Socs.*, 1914, ix, 186.
15. Marshall, J. A., *Am. J. Physiol.*, 1914–15, xxxvi, 260.
16. Marshall, J. A., *Dent. Items of Interest*, 1916, xxxviii, 116.
17. Marshall, J. A., *Am. J. Physiol.*, 1916, xl, 1.
18. Marshall, J. A., *Dent. Cosmos*, 1916, lviii, 1225.
19. Marshall, J. A., *Dent. Cosmos*, 1917, lix, 33.
20. Ludlum, S. D., *Med. Clin. of North America*, 1918, ii, 3, 895.
21. Gies, W. J., *J. Allied Dent. Socs.*, 1911, vi, 334.
22. Gies, W. J., and Lothrop, *J. Allied Dent. Socs.*, 1911, vi, 65.
23. Gies, W. J., *J. Allied Dent. Socs.*, 1914, ix, 346.
24. Gies, W. J., *J. Allied Dent. Socs.*, 1916, xi, 273.
25. Gies, W. J., and Shepard, L. A., *J. Allied Dent. Socs.*, 1916, xi, 275.
26. Gies, W. J., *J. Allied Dent. Socs.*, 1916, xi, 488.

27. Gies, W. J., *J. Allied Dent. Socs.*, 1916, xi, 659.
28. Lowenstein, G. A., and Gies, W. J., *Proc. Soc. Exp. Biol. and Med.*, 1918–19, xvi, 53.
29. Pickerill, H. P., The prevention of dental caries and oral sepsis, Philadelphia, 1st edition, 1910; New York, 2nd edition, 1919.
30. Prinz, H., *Dent. Cosmos*, 1918, lx, 140.
31. Prinz, H., *Dent. Cosmos*, 1918, lx, 197.
32. Prinz, H., *Dent. Cosmos*, 1918, lx, 287.
33. Sullivan, M. X., and Jones, K. K., *Pub. Health Rep. 526*, 1919, 3.
34. Sullivan, M. X., and Dawson, P. R., *J. Biol. Chem.*, 1920–21, xlv, 473.
35. Heidenhain, R., in Hermann, L., Handbuch der Physiologie, 1883, v, pt. 1.
36. Langley, J. N., *J. Physiol.*, 1889, x, 291, 433.
37. Pawlow, J. P., The work of the digestive glands, London, 1902.
38. Cannon, W. B., Bodily changes in pain, hunger, fear, and rage, New York and London, 1915.
39. Chittenden, R. H., and Ely, J. S., *Am. Chem. J.*, 1882–83, iv, 329.
40. Chittenden, R. H., and Smith, H. E., *Tr. Connecticut Acad. Arts and Sc.*, 1882–85, vi, 343.
41. Schlesinger, A., *Virchows Arch. path. Anat.*, 1891, cxxv, 146, 340.
42. Szabo, J., *Ungar. med. Presse*, 1900, v, 653.
43. Cohn, M., *Deutsch. med. Woch.*, 1900, xxvi, 68.
44. Foa, C., *Arch. fisiol.*, 1905–06, iii, 369.
45. Michaelis, L., and Pechstein, H., *Biochem. Z.*, 1914, lix, 77.
46. Graham, L. W., *Dent. Cosmos.*, 1919, lxi, 409.
47. Bloomfield, A. L., and Huck, J. G., *Bull. Johns Hopkins Hosp.*, 1920, xxxi, 118.
48. Sørensen, S. P. L., *Ergebn. Physiol.*, 1912, xii, 398.
49. Henderson, L. J., and Palmer, W. W., *J. Biol. Chem.*, 1912–13, xiii, 398.
50. Clark, W. M., The determination of hydrogen ions, Baltimore, 1920.
51. Van Slyke, D. D., Stillman, E., and Cullen, G. E., *J. Biol. Chem.*, 1919, xxxviii, 167.
52. Cullen, G. E., *J. Biol. Chem.*, 1922, lii, 501.

STUDIES OF HUMAN MIXED SALIVA.

II. VARIATIONS IN THE HYDROGEN ION CONCENTRATION OF HUMAN MIXED SALIVA.

By HENRY E. STARR.

(From the Robert Hare Laboratory of Chemistry, School of Medicine, and the Psychological Laboratory and Clinic, University of Pennsylvania, Philadelphia.)

The fact that human mixed saliva loses CO_2 very readily and increases in pH, as noted in the preceding article in this series, indicates the importance of the $H_2CO_3:BHCO_3$ ratio[1] in determining the hydrogen ion concentration. This is quite in accord with the findings of various quantitative investigations of salivary acidity. Thus Dieminger (2) concluded that saliva will always be found alkaline if one uses an indicator insensitive to carbon dioxide. In 1914, Gies (3) pointed out that aerating saliva causes a decomposition of soluble bicarbonate with elimination of carbon dioxide, so that the original reaction of the saliva cannot be accurately determined after aeration.[2]

Study of the $H_2CO_3:BHCO_3$ ratio in mixed saliva at various hydrion concentrations and alveolar CO_2 tensions, etc., and of the base content of the saliva is now in progress and will be reported

---

[1] Terminology used by Van Slyke (1). The use of B as the general symbol of a univalent base, rather than the formerly indiscriminately applied "Na" is particularly useful in the case of human mixed saliva, in which there is three to five times as much K as Na.

[2] In the earlier stages of the present research, CO_2-free air was passed successively through saliva to which an indicator had been added (bromthymol blue) and then through standard $Ba(OH)_2$ solutions. The amount of CO_2 absorbed by the latter was determined by titration and the change in pH of the saliva was also noted. It was found that increase in pH of the aerated saliva and elimination of CO_2 took place *pari passu*. The violent agitation of the saliva during aeration precluded precipitation of any acidic sediment and consequently the decrease in acidity was attributable to loss of CO_2.

in this series. As a result of a preliminary survey, involving a number of simultaneous determinations of salivary pH and of total CO_2 content of the saliva, made by means of the Van Slyke apparatus, in the standard manner, it is evident that the hydrion concentration of the mixed saliva is largely determined by the H_2CO_3 content of the saliva.

Influence of Alveolar CO_2 on Salivary pH.

To determine whether the salivary pH varies inversely with the alveolar CO_2 simultaneous determinations of salivary pH and alveolar CO_2 were made at intervals during days of mixed diet and normal activity on five healthy and normal subjects. In order to insure making determinations when the alveolar CO_2 content varied, specimens were taken before and after the noon meal, inasmuch as Van Slyke, Stillman, and Cullen (4) have confirmed the findings of Higgins (5) and others that digestive activity is accompanied by a rise in alveolar CO_2. Prior to ejection of the saliva, the mouth was kept closed for 5 minutes, the saliva was then collected under oil and its pH determined as described in the preceding article. The sample of alveolar air was taken immediately after ejection of the saliva, and the CO_2 content was determined by the Fridericia method (6), care being taken by the subjects to avoid either holding the breath or taking a preliminary deep inspiration. The results are given in Table I, and a typical series is shown in Fig. 1.

The findings presented in Table I and Fig. 1 indicate a close correlation between salivary hydrion concentration and alveolar CO_2.

Alkali Retention.

As a further test along lines similar to the above, a modification of the alkali retention test was employed. The subject was examined as to salivary and urinary pH and then immediately ingested 10 to 20 gm. of $NaHCO_3$, with a copious draught of water. At intervals the pH determinations were repeated.[3] During the course of the experiment, no food was eaten, water was taken as desired, the subjects were normally active, the environment quite

---

[3] Both saliva and urine were collected and kept under oil during the pH determinations.

calm, and consequently the effect of the ingestion of the NaHCO$_3$ could be observed without hindrance. The experiment was repeated ten times with four healthy normal subjects. In every instance it was found that as the urinary pH increased, the salivary pH decreased. A typical series is shown in Fig. 2.

TABLE 1.

Variations in Salivary pH and Alveolar CO$_2$ after Noon Meal.

No.	Subject.	Time in relation to meal.	Salivary.	Alveolar CO$_2$.
			pH	*per cent*
1	IFT	20 min. before.	6.75	5.50
		40 " after.	6.60	5.70
2	IFT	20 " before.	6.60	5.75
		40 " after.	6.50	6.05
3	IG	20 " before.	6.65	6.00
		40 " after.	6.50	6.05
4	KH	20 " before.	6.25	6.45
		60 " after.	6.00	6.75
		90 " "	6.15	6.50
5	GXT	20 " before.	6.75	5.45
		40 " after.	6.55	6.05
6	KKs	20 " before.	6.35	6.10
		40 " after.	6.40	6.10
7	IFT	20 " before.	6.55	5.95
		40 " after.	6.50	6.05
		60 " "	6.45	6.15
		90 " "	6.35	6.50
		120 " "	6.50	6.10

The results show that after the ingestion of the bicarbonate, the H ion concentration of the mixed saliva increased while that of the urine decreased; *i.e.*, the salivary pH varied inversely with that of the urine in every instance. Thus while the bodily fluids in general may be regarded as becoming more "alkaline" as the result of the administration of sodium bicarbonate, the mixed saliva became more "acid."

FIG. 1. Variations of salivary pH with alveolar CO_2 (Experiment 7, Table I).

FIG. 2. The effect of the ingestion of 20 gm. of $NaHCO_3$ upon salivary and urinary pH.

These findings tend to explain and reconcile the findings of two investigators in different biochemic fields. Von Noorden (7) found that removal of carbohydrates from the diet of an individual is usually followed within 24 hours by a definite "physiological acidosis" with characteristic excretion of acetone bodies. Kirk (8) found that the removal of carbohydrates from the diet of certain patients tended to decrease the "acidity" of the saliva. The explanation may be that lack of carbohydrate ingestion may result in an acetone body acidosis with a decrease in the blood $BHCO_3$, followed by a compensatory drop in the blood H_2CO_3 and alveolar CO_2 resulting in a more alkaline saliva.

Voluntary Deep Breathing.

It should be recalled in this connection, as Van Slyke (9) has pointed out, as regards the hydrion concentration of the blood expressed in terms of pH that "probably the extreme range compatible with life is 7.0 to 7.8. The normal range is possibly somewhat narrower than ·7.3 to 7.5," and that "by voluntary deep breathing, CO_2 may be blown off until blood alkalinity rises to a pH of 7.7 or 7.8 (Davis, Haldane, and Kennaway, 1920; Collip and Backus, 1920) at which point symptoms of tetany appear."

To determine the effect of such "voluntary deep breathing" upon salivary pH, nine subjects were placed out of doors immediately after a salivary pH determination had been made in each case, and were required to inhale vigorously and deeply and exhale forcefully, keeping the mouth tightly closed, for 10 minutes, at the expiration of which time the salivary pH was again determined. The results are given in Table II including those obtained from IFT after ingestion of 15 gm. of sodium bicarbonate, indicated in the table by the asterisk (*).

Table II shows clearly that voluntary deep breathing in the open air for 10 minutes results in increase in salivary pH. The increases noted ranged from 0.15 to 1.15. It is evident, therefore, that any interpretation of salivary pH findings must take into consideration the breathing habits of and the air breathed by the subject.

The factors discussed above, in addition to uncontrolled loss of CO_2, probably furnish the explanation of the divergencies and

contradictions in the reports of various investigators of the "acidity" of mixed saliva in various dental conditions, which have led some to regard an acid and others an alkaline saliva as indicative, and indeed causative, of the same dental disease. In 1916, Gies (10) stated that he had concluded it to be "biologically absurd to deduce anything of diagnostic dental import from the nature and degree of reaction of any specimen or any pair of specimens, of saliva from anybody under any conditions." This conclusion is sweeping, but the explanation is no doubt

TABLE II.

The Effect of Voluntary Deep Breathing upon Salivary pH.

Subject.	Reaction of saliva.		
	Initial.	After 10 min. deep breathing.	Increase.
	pH	pH	pH
KH	6.75	6.90	0.15
GQ	6.70	6.85	0.15
T	6.75	6.95	0.20
IFT	6.65	6.85	0.20
1G	6.50	6.75	0.25
GXT	6.85	7.10	0.25
KN	6.40	6.70	0.30
KKs	6.80	7.30	0.50
BMT	6.25	6.85	0.60
IFT*	5.80	6.95	1.15

*After the ingestion of 15 gm. of sodium bicarbonate.

found in the simple fact that breathing conditions, probably through their effect upon the carbon dioxide tension of the blood and of the alveolar air, determine largely the free carbon dioxide content of the mixed saliva and thereby its hydrion concentration.

Diurnal Rhythm in Salivary pH during Fatigue and Excitement.

The findings of Chittenden and Richards (11), of Cohn (12), and of van der Molen and Offringa (13) indicated a diurnal rhythm in salivary alkalinity. It appeared not unlikely that there might be found a diurnal rhythm in salivary pH. Accordingly salivary pH determinations were made on a subject at half hour

intervals from 7.00 a.m. to 6.00 p.m. on a day of mixed diet and inactivity, on a day of mixed diet and normal activity, on a day of abstinence from food and inactivity, and on a day of abstinence from food and normal activity. The results indicated a drop in pH soon after the ingestion of food, followed by a rise except during an afternoon of fairly strenous activity when there was a steady fall during the afternoon working hours. Nine similar determinations were made on seven other subjects on days of mixed diet and normal working activity. They also showed decreasing salivary pH with increasing fatigue. During this investigation four of the subjects were emotionally excited at the time of the collection of a specimen of saliva—one by fear and three by anger. In each instance the salivary pH showed a sharp rise during excitement and a subsequent lowering of salivary pH as the excitement subsided. The increases noted were 0.60, 0.45, 0.50, and 0.20 pH during excitement.

Salivary pH of Normal Subjects and Stammerers.

These findings were discussed with Dr. E. B. Twitmyer, Director of the Clinic for Speech Defects at this university. In his examination of stammerers for many years, he has found that a large number of them have so little chest expansion or are such shallow breathers that they may be termed "sub-breathers." Often dull and lethargic, many appear to be chronically fatigued. Another type of stammerer which he has noted is the hyperexcitable psychopath. It appeared probable that the "sub-breathers" would show a low salivary pH and the chronically excited psychopaths, a high salivary pH. Accordingly pH determinations were made upon 610 specimens of saliva from 228 healthy normal individuals, 200 specimens from 58 typical "sub-breathing" stammerers, and 50 specimens from 10 distinctly psychopathic stammerers. In every instance the mouth was kept closed for a period of 5 minutes during which the saliva was allowed to collect naturally. It was then ejected and its pH immediately determined.[4] The results expressed in terms of relative frequency are shown in the form of a triple graph in Fig. 3.[4]

[4] For a detailed report of the work on stammerers, including the psychological technique, interpretations, etc., see Starr, H. E., *Am. J. Psychol.*, 1922, xxxiii, 394.

Fig. 3 indicates that the salivary pH of the sub-breathers as a class is distinctly below, and that of the psychopaths distinctly above, that of the normal individual. The range of pH values for normal individuals was found to be from 5.75 to 7.05. The mean was 6.6, the median 6.6, and the mode 6.6 to 6.7 inclusive. 86 per cent of the specimens ranged from 6.35 to 6.80 inclusive.

Emotional Excitation.

As a further test of correlation between salivary pH and emotional excitement, the following procedure was adopted. The salivary pH of a subject was determined. He was then subjected to an emotionalizing stimulus, carefully standardized, of such character that it produced no effect upon either a dull or normally stable individual, but was quite sufficient to disturb the unstable equilibrium of a hyperexcitable subject. After 5 minutes of such stimulation, during which he was instructed to

TABLE III.

The Effect of an Emotionalizing Stimulus upon Salivary pH.

Subjects (stammerers).	Average initial pH.	Average increase above initial pH after	
		5 min. stimulus.	10 min. rest.
	pH	*pH*	*pH*
Lethargic..........................	5.80	0.04	0.05
Excitable..........................	7.24	0.40	0.12

keep his mouth closed, the salivary pH was again determined. He was then allowed a 10 minute rest period, after which a third salivary pH determination was made. Each time a specimen of saliva was taken, a clinical diagnosis of the subject's emotional condition was made and recorded. Twenty-seven "sub-breathers" and ten psychopaths were thus examined. The results are summarized in Table III and tend to confirm the conclusion that emotional excitement is accompanied by increase in salivary pH.

Fig. 3 is adapted from Charts 1 and 2 in the above, the values having been corrected to approximate more closely the actual salivary pH, however, inasmuch as Method B was employed in making these determinations.

Fig. 3. Relative frequency of salivary pH values.

CONCLUSIONS.

1. The hydrogen ion concentration of human mixed saliva has been found to vary directly with the alveolar CO_2 after the noon meal. It was also found to vary inversely with the hydrion concentration of the urine after the ingestion of large doses of $NaHCO_3$. Vigorous forced breathing in the open air, with mouth closed, resulted in decreased acidity of the mixed saliva.

2. The $[H^+]$ of the mixed saliva was found to vary inversely with the degree of energy displayed by an individual, increasing during fatigue and decreasing during emotional excitement.

3. A group of stammerers who were habitually deficient in the use of their lungs and lethargic in behavior, showed a characteristically high salivary $[H^+]$. A group of hyperexcitable psychopathic stammerers showed an equally characteristic low salivary $[H^+]$.

4. 610 specimens of human mixed saliva collected from 228 healthy normal subjects varied in hydrogen ion concentration from pH 5.75 to 7.05. 86 per cent of the specimens were between pH 6.35 and 6.80 inclusive. The mean, mode, and median practically coincided at about pH 6.60.

5. These observations indicate that the $[H^+]$ of the mixed saliva parallels the alveolar CO_2 and the H_2CO_3 content of the blood, rather than the $[H^+]$ of the blood.

BIBLIOGRAPHY.

1. Van Slyke, D. D., *Physiol. Rev.*, 1921, i, 141.
2. Dieminger, H., Beiträge zur Kenntniss des menschlichen Mundspeichels in gesunden und pathologischen Verhältnissen, Dissertation, Würzburg, 1898.
3. Gies, W. J., *J. Allied. Dent. Socs.*, 1914, ix, 346,
4. Van Slyke, D. D., Stillman, E., and Cullen, G. E., *J. Biol. Chem.*, 1917, xxx, 401.
5. Higgins, H. L., *Am. J. Physiol.*, 1914, xxxiv, 114.
6. Fridericia, L. S., *Berl. klin. Woch.*, 1914, li, 1268.
7. Von Noorden, cited in Hawk, P. B., Practical physiological chemistry, Philadelphia, 7th edition, 1922, 305.
8. Kirk, E. C., *Dent. Rev.*, 1903, xvii, 383.
9. Van Slyke, D. D., *J. Biol. Chem.*, 1921, xlviii, 153.
10. Gies, W. J., *J. Allied Dent. Socs.*, 1916, xi, 188.
11. Chittenden, R. H., and Richards, A. N., *Am. J. Physiol.*, 1898, i, 461.
12. Cohn, M., *Deutsch. med. Woch.*, 1900, xxvi, 68.
13. van der Molen, H., and Offringa, J., *Biochem. Z.*, 1909, xv, 350.

THE STRUCTURE OF FUCOSE.

By E. P. CLARK.

(*From the Polarimetry Section, United States Bureau of Standards, Washington.*)

Some relationships existing between the constitution and optical rotatory powers of certain sugar derivatives have been discovered by Hudson and by Levene. Hudson (1) found that when the formula of a sugar acid lactone, *i.e.*, a gamma lactone, is written vertically thus,

the compound will rotate to the right when the lactone ring is on the right of the carbon chain; and, *vice versa*, when the ring is on the left of the chain, the compound will be levo-rotatory. As the position of the ring is determined by the position which the hydroxyl group had on the carbon atom 4 before the ring was formed, a means of determining the position of the hydroxyl group on this carbon atom is thus available. He also found (2) that the dextro-rotatory amides of the sugar acids have the hydroxyl group on carbon atom 2 on the right of the carbon chain, while the levo-rotatory amides have the hydroxyl on the left. Levene (3) found that the same principle holds for metallic salts and phenylhydrazides of the sugar acids.

65

Now the configuration of fucose has been established (4) to be that of a methyl pentose,

$$
\begin{array}{ll}
\mathrm{H} \diagdown \quad \mathrm{O} \\
\quad \underset{|}{\mathrm{C}} \diagup\!\!\diagup & 1 \\
\mathrm{HO-}\underset{|}{\mathrm{C}}-\mathrm{H} & 2 \\
\mathrm{H-}\underset{|}{\mathrm{C}}-\mathrm{OH} & 3 \\
\mathrm{H-}\underset{|}{\mathrm{C}}-\mathrm{OH} & 4 \\
\underset{|}{\mathrm{C}}\ \ \mathrm{HOH} & 5 \\
\mathrm{C}\ \ \mathrm{H_3}
\end{array}
$$

the position of the hydroxyl group on carbon atom 5 not having yet been determined. It would follow from the above that if a methyl tetronic acid lactone could be obtained from fucose, it would be possible to apply the above rule to it and thus determine the position of the hydroxyl group on carbon atom 4 of the lactone, which corresponds to carbon atom 5 in fucose. Previous to the present investigation this compound had not been prepared; but Hudson and Chernoff (5) obtained a methyl tetronic acid lactone from rhamnose by applying a method devised by Nef, Hedenburg, and Glattfeld (6), for the oxidation of pentoses to the corresponding tetronic acids. The same results should be expected from fucose, giving the methyl tetronic lactone desired. This proved to be the case. A yield of 7.5 gm. of the substance was obtained from 50 gm. of fucose. The lactone was strongly levo-rotatory ($-63.65°$), which indicates that its configuration is

$$
\begin{array}{ll}
\quad\ \ \underset{\|}{\mathrm{O}} \\
\ \mathrm{-C} & 1 \\
|\quad -\underset{|}{\mathrm{C}}- & 2 \\
\mathrm{O}\quad\ |\\
|\quad -\underset{|}{\mathrm{C}}- & 3 \\
\ \mathrm{-C-} & 4 \\
\qquad \underset{|}{\mathrm{C}}\ \ \mathrm{H_3}
\end{array}
$$

and hence, the configuration of fucose is

$$
\begin{array}{cc}
\text{H} \diagdown \quad \diagup \text{O} & \\
\quad\;\; \text{C} & 1 \\
\quad\;\; | & \\
\text{HO}-\text{C}-\text{H} & 2 \\
\quad\;\; | & \\
\text{H}-\text{C}-\text{OH} & 3 \\
\quad\;\; | & \\
\text{H}-\text{C}-\text{OH} & 4 \\
\quad\;\; | & \\
\text{HO}-\text{C}-\text{H} & 5 \\
\quad\;\; | & \\
\quad\;\; \text{C}\;\;\text{H}_3 &
\end{array}
$$

This is in accordance with the conclusion reached by Mayer and Tollens (7), based upon the similarity of the specific rotation of *l*-galactose ($-81°$) and fucose ($-75.5°$).

Incidentally, the position of the other three hydroxyl groups in fucose may be verified by the same line of reasoning. Fuconic acid lactone (8) is dextro-rotatory ($+71.7°$); therefore, the hydroxyl on the carbon atom 4 will, according to the rule, be on the right of the carbon chain. Fuconic amide, on the other hand, as will be shown in the experimental part, is levo-rotatory ($-31.13°$); hence, the position of the hydroxyl group on carbon atom 2 is on the left of the carbon chain. The amide of the methyl tetronic acid was prepared, as described below, so that the position of the hydroxyl group on carbon atom 2 in this compound, corresponding to carbon atom 3 in fucose, could be verified. This substance was found to be dextro-rotatory ($+18.48°$); therefore, the hydroxyl group on this carbon atom is on the right of the chain. Its position is further established as Hudson has pointed out (4) by the fact that the methyl hexonic lactone prepared by Mayer and Tollens from fucose (9) is dextro-rotatory ($+33.3°$). It will be seen from the formula that the carbon atom 2 in the methyl tetronic acid becomes carbon atom 4 in either of the two fucohexonic acids.

Thus, by the preparation of the methyl tetronic acid lactone from fucose and the application of the above rules to this substance, the position of the hydroxyl group on the carbon atom 5 of fucose has been determined. Likewise, the positions of the

hydroxyl groups on carbon atoms 2, 3, and 4 of this sugar, heretofore determined by purely chemical means, have been verified.

The present communication also contains an improved method for the preparation of fucose.

Preparation of Fucose.

About 10 kilos of seaweed,[1] which has been air-dried sufficiently to become brittle, are coarsely ground in a meat chopper. The ground material is then treated, at room temperature for 2 days, with 3 per cent HCl (3 liters to each kilo), after which it is repeatedly washed with water by decantation until the washings are no longer acid to litmus. The residue is pressed out and dried.

1 kilo of the dried substance is added to 8 liters of boiling 2 per cent sulfuric acid and simmered for 3 hours. The undissolved material is filtered upon large Buchner funnels and thoroughly washed. The sulfuric acid in the filtrate and washings is neutralized with a paste of precipitated barium carbonate. The resulting barium sulfate is removed by filtering the solution through a thin layer of decolorizing carbon on moistened filter paper in a Buchner funnel. The filtrate is treated with basic lead acetate until no further precipitate is formed. The solution is next freed from the lead precipitate, and the excess of lead in the filtrate removed by the cautious addition of dilute sulfuric acid. After the removal of the lead sulfate the solution is evaporated under diminished pressure to a syrup of about 175 cc. This syrup is dissolved in an equal volume of methyl alcohol by warming on a water bath and sufficient ethyl alcohol added to make 2 liters. The precipitate formed is filtered upon decolorizing carbon, as outlined above, and the filtrate evaporated to 150 cc. The syrup is washed from the flask with small portions of absolute alcohol by warming, and, when all is removed, sufficient absolute alcohol is added until the solution has a volume of 450 cc. 75 gm. of phenylhydrazine are added and the mixture is placed in

[1] The material used in this work was *Ascophyllum nodosum*, which was obtained from Woods Hole, Mass., through the courtesy of Dr. R. E. Coker of the United States Bureau of Fisheries.

an ice box over night to crystallize completely. The hydrazone is filtered and washed, first with absolute alcohol and finally with ether. The yield is 100 gm.

To obtain the sugar, the hydrazone thus prepared is sufficiently pure. 75 gm. of the material are suspended in 1,800 cc. of water which is heated to about 90°C. and 36 gm. of benzaldehyde are added while the mixture is rapidly stirred. The stirring is continued for $\frac{1}{2}$ to $\frac{3}{4}$ of an hour. It is advantageous to have an atmosphere of carbon dioxide in the flask to prevent the benzaldehyde from becoming oxidized. After removing the benzaldehyde phenylhydrazone the filtrate is treated with carbon. The fucose solution is then concentrated to a thick syrup and dissolved in 800 cc. of hot absolute alcohol. Some impurities are thrown out by this process, so the solution is filtered, after which it is concentrated to about 50 cc. This syrup is removed to a beaker and the flask washed out with small portions of absolute alcohol by warming, 50 cc. in all being used for this purpose. The sugar begins to crystallize almost at once, and the crystallization is completed over night in an ice box. It is freed from mother liquor, washed with absolute alcohol and ether, and dried. The yield is 38 to 40 gm. A sample of this material had the following rotation:

$$[\alpha]_D^{20} = -75.55° \ (2.5015 \text{ gm. dissolved in 50 cc.}).$$

Generally, another small crop of crystals may be obtained by concentrating the mother liquors.

To recrystallize the sugar it is dissolved in distilled water making a 25 per cent solution and treated with decolorizing carbon. The filtrate is concentrated under diminished pressure to a thick syrup and dissolved from the flask, by warming on the water bath, with 3 volumes of absolute alcohol, then allowed to crystallize. The crystals are washed with absolute alcohol and ether, and dried. The mother liquors may be worked over so that almost a quantitative yield is obtained. The purified sugar had the following specific rotation:

$$[\alpha]_D^{20} = -75.48° \ (5.0742 \text{ gm. of sugar in 100 cc.}).$$

$$[\alpha]_{\lambda\,5461\,\text{Å. u.}}^{20} = -88.92° \ (5.0742 \text{ gm. of sugar in 100 cc.}).$$

Methyl Tetronic Acid Lactone from Fucose.

50 gm. of fucose were dissolved in 250 cc. of water and added to a solution of 93 gm. of KOH dissolved in 5,250 cc. of water. Air freed from carbon dioxide was continuously drawn through the mixture at a fairly rapid rate for 60 hours by means of a water pump. During working hours the solution was heated to 40 to 45°. The KOH was then neutralized and the organic acids liberated by the cautious addition of HCl. This was determined by means of thymol blue. Just enough HCl was added to give a distinct red color, showing the presence of free HCl. The solution was evaporated under diminished pressure until a fairly large amount of KCl had separated. This was filtered off and washed with a little cold water and the filtrate adjusted to the point where all the free HCl was exactly neutralized. Concentration was continued to almost dryness, and the resulting mass dissolved in a little water, and again evaporated to dryness to insure the removal of formic acid.

The residue was extracted three times with boiling absolute alcohol, 250 cc. being used for each extraction. The alcoholic liquor was filtered and evaporated to a thick syrup, dissolved in about 100 cc. of absolute alcohol, and again evaporated. The resulting syrup was extracted with 500 cc. of dry ethyl acetate. An insoluble gum remained, while the supernatant liquid was cloudy; but, after a day, an insoluble gum separated leaving a clear solution. This solution was evaporated under diminished pressure to a mobile liquid which was exhausted with dry ether by vigorous shaking for some time. After a day the ether layer was clear and a gum-like substance separated. Upon standing 2 days more, beautiful, long crystals appeared on the walls of the flask while the insoluble material was a semisolid mass of crystals. The ether was decanted and the crystals were dissolved by warming in 30 cc. of ethyl acetate. Upon cooling, the solution immediately crystallized. The yield of the crude product was 7.5 gm. The substance was removed from the mother liquor and recrystallized from a few cc. of ethyl acetate.

Its melting point was 111°C., uncorrected. The substance when first dissolved in water was neutral, but gradually became acid, showing its lactone character. 0.0928 gm. of the material was dissolved in 10 cc. of

0.1 N KOH, warmed to the temperature of a boiling water bath, and allowed to cool. The excess of KOH was titrated with 0.1 N HCl using phenolphthalein as an indicator. In this way the sample required 7.01 cc. of 0.1 N KOH for neutralization; calculated for $C_5H_8O_4$, 7.03 cc. The optical rotation of the substance was as follows: 1.2529 gm. dissolved in 25 cc. of water at 20°C. rotated the plane of polarized light 6.38° to the left in a 2 dm. tube using sodium light. Therefore,

$$[\alpha]_D^{20} = \frac{(100)(-6.38)}{4(1.2529)(2)} = -63.65°$$

0.2028 gm. of substance gave on combustion 0.1101 gm. of water and 0.3379 gm. of CO_2.

$C_5H_8O_4$.　Calculated.　　C 45.43, H 6.10.
　　　　　　Found.　　　" 45.44, " 6.07.

Methyl Tetronic Amide.

Dry ammonia was passed into a solution of 3.3 gm. of the lactone dissolved in 30 cc. of absolute alcohol until it became saturated. Crystallization of the amide could not be induced; so after a day, the liquid was evaporated under diminished pressure to about 10 cc., and 10 cc. of dry ether were added. After a week, crystallization commenced and was complete in a very short time after it started. The crystals were filtered, washed with absolute alcohol and ether, and dried. The yield was 2.5 gm. The melting point of the crude substance was 112°, uncorrected. To recrystallize the compound, it was dissolved, by warming, in 15 cc. of absolute alcohol, filtered, and 15 cc. of dry ether were added.

The melting point was 112.5°. Its specific rotation was as follows: 0.5006 gm. of substance in water to make 25 cc. rotated the plane of polarized light 0.74° to the right, using a 2 dm. tube; hence,

$$[\alpha]_D^{20} = \frac{(100)(+0.74°)}{(4)(0.5006)(2)} = +18.48°$$

Nitrogen was determined by the Kjeldahl method.
0.2537 gm. of substance used 17.07 cc. of 0.1 N HCl.
$C_5H_{11}O_4N$.　Calculated.　　N 9.40.
　　　　　　Found.　　　" 9.43.

Fuconic Amide.

This substance has not hitherto been recorded. 15 gm. of fucose were dissolved in 75 cc. of water and 15 gm. of bromine

added. Upon shaking occasionally, during 1 hour, the bromine was dissolved. The solution was allowed to stand for 2 days, after which the excess of bromine was removed by distillation under reduced pressure. The resulting liquid was made neutral with sodium hydroxide and then treated with slightly more than the calculated amount of barium chloride. Barium fuconate immediately began to crystallize, the crystallization being complete as soon as the solution cooled to room temperature. The yield was 19.5 gm. The barium was removed from 10 gm. of this salt by the addition of sulfuric acid. The resulting solution was freed from barium sulfate and evaporated under diminished pressure to a thick syrup, after which it was heated, *in vacuo*, at the temperature of a boiling water bath for $\frac{1}{2}$ hour. The residue was dissolved, by warming, in 150 cc. of absolute alcohol, and filtered. The filtrate was evaporated to a thick syrup, dissolved in 100 cc. more of absolute alcohol and again evaporated to a syrup. This was dissolved in 60 cc. of absolute alcohol and the solution saturated with dry ammonia. Crystallization of the amide began within a few minutes.

The melting point of the crude product was 180.5°, uncorrected. Upon recrystallizing from 85 per cent alcohol its melting point was unchanged.

1.0021 gm. of substance in water to make 50 cc. rotated the plane of polarized light 1.248° to the left, using a 2 dm. tube; hence,

$$[\alpha]_D^{20} = \frac{(100)\,(-1.248)}{2\,(1.0021)\,(2)} = -31.13°$$

By Kjeldahl estimation 0.3037 gm. of substance used 16.86 cc. of 0.1 N HCl.

$C_6H_{13}O_5N$.   Calculated.   N 7.82.
               Found.   " 7.78.

SUMMARY.

1. An improved method for the preparation of fucose is described.

2. The methyl tetronic acid lactone from fucose has been prepared, and from its optical properties the position of the hydroxyl group on carbon atom 5 of fucose has been determined, thus giving the complete structure of fucose.

3. The amides of the methyl tetronic acid and fuconic acid have been prepared, and from a study of their optical properties,

together with those of fuconic lactone, the positions of the hydroxyl groups on carbon atoms 2, 3, and 4 of fucose, heretofore determined by purely chemical means, have been verified.

BIBLIOGRAPHY.

1. Hudson, C. S., *J. Am. Chem. Soc.*, 1910, xxxii, 338.
2. Hudson, C. S., *J. Am. Chem. Soc.*, 1918, xl, 813.
3. Levene, P. A., *J. Biol. Chem.*, 1915, xxiii, 145. Levene, P. A., and Meyer, G. M., *J. Biol. Chem.*, 1916, xxvi, 355; 1917, xxxi, 623.
4. Günther, A., and Tollens, B., *Ann. Chem.*, 1892, cclxxi, 86. Müther, A., and Tollens, B., *Ber. chem. Ges.*, 1904, xxxvii, 306. Votoček, E., *Ber. chem. Ges.*, 1904, xxxvii, 3859; 1910, xliii, 469. Mayer, W., and Tollens, B., *Ber. chem. Ges.*, 1907, xl, 2434. Tollens, B., and Rorive, F., *Ber. chem. Ges.*, 1909, xlii, 2009. Hudson, C. S., *J. Am. Chem. Soc.*, 1911, xxxiii, 405.
5. Hudson, C. S., and Chernoff, L. H., *J. Am. Chem. Soc.*, 1918, xl, 1005.
6. Nef, J. U., Hedenburg, O. F., and Glattfeld, J. W. E., *J. Am. Chem. Soc.*, 1917, xxxix, 1638.
7. Mayer, W., and Tollens, B., *Ber. chem. Ges.*, 1907, xl, 2440.
8. Müther, A., and Tollens, B., *Ber. chem. Ges.*, 1904, xxxvii, 308.
9. Mayer, W., and Tollens, B., *Ber. chem. Ges.*, 1907, xl, 2436.

DIETARY FACTORS INFLUENCING CALCIUM ASSIMILATION.

III. THE COMPARATIVE EFFICIENCY OF TIMOTHY HAY, ALFALFA HAY, AND TIMOTHY HAY PLUS CALCIUM PHOSPHATE (STEAMED BONE MEAL) IN MAINTAINING CALCIUM AND PHOSPHORUS EQUILIBRIUM IN MILKING COWS.*

By E. B. HART, H. STEENBOCK, C. A. HOPPERT, AND R. M. BETHKE,

(From the Department of Agricultural Chemistry, University of Wisconsin, Madison.)

AND G. C. HUMPHREY.

(From the Department of Animal Husbandry, University of Wisconsin, Madison.)

In a recent publication (1) we reported evidence secured with liberally milking cows showing that it was possible to maintain calcium and phosphorus equilibrium with an alfalfa hay *which had been cured under caps* and with dried grains and their concentrated by-products such as wheat bran. The alfalfa served as the principal source of calcium in the ration and the grains and wheat bran as the principal source of phosphorus. These results we believe are of some importance to the dairy industry, although they are not in harmony with observations of Forbes (2), who found constant negative calcium balances in milking cows receiving alfalfa hay, nor with observations recorded in this paper which were obtained with an alfalfa hay cured in the *windrow for 4 days with exposure to air and light.*

In these later experiments we have extended our observations to include calcium and phosphorus balances on lactating cows receiving in succeeding periods, respectively, timothy hay, al-

* Published with the permission of the Director of the Wisconsin Agricultural Experiment Station.

falfa hay, and timothy hay plus calcium phosphate, as steamed bone meal, in addition to corn silage and a grain mixture. We did this in order to secure further data on the effectiveness of alfalfa hay in maintaining calcium equilibrium in a liberally milking animal. We felt certain that timothy hay grown on an acid soil and containing but 0.4 per cent of calcium oxide would, even when supplemented by corn silage and a proper grain mixture, lead to a negative calcium balance with milking cows. Further, if the negative calcium balance obtained on the timothy hay could be changed to a positive calcium balance by substituting alfalfa hay for the timothy hay the effective properties in respect to calcium assimilation of alfalfa in general and alfalfa in a dried state would be more firmly established. We also wanted to know how efficient calcium phosphate, added to timothy hay, would be in maintaining calcium equilibrium. The amount of calcium phosphate added was such as to make the intake of calcium approximately the same as when the animals received alfalfa hay.

EXPERIMENTAL.

We used three animals for this work. No. 1 was a pure-bred Holstein; No. 2, a pure-bred Guernsey; and No. 3, a grade Holstein. Their weights at the beginning and end of the experimental period were, No. 1, 1,234 to 1,166 pounds; No. 2, 1,185 to 1,096 pounds; No. 3, 1,119 to 1,119 pounds. They were all fresh milkers. No. 1 had freshened in November, 1921; No. 2, in September, 1921; and No. 3, in November, 1921. This experiment was begun December, 1921.

These animals were confined to metabolism stalls for a total period of 13 weeks with quantitative collection of the excreta by men working in three 8 hour shifts. None of the animals was with calf although they were all bred during the experimental period.

A definite daily aliquot of feces, urine, and milk was composited for a period of 7 days and analyses for phosphorus and calcium were made upon these composite samples. The feces were dried and finally analyzed in an air-dried condition. Phosphorus was determined in the urine by the Neumann method, and in the feeds, feces, and milk after ashing with magnesium nitrate. Calcium was always determined by McCrudden's method.

The feeds used in Period 1, which was for 4 weeks, were a timothy hay grown on an acid soil, corn silage, and a grain mixture consisting of 60 parts of yellow corn, 15 parts of oil meal, and 25 parts of wheat bran. The proportions used were 10 pounds of hay for each animal per day; 20 to 25 pounds of silage per day; and approximately 1 pound of the grain mixture for 3 pounds of milk produced per day. Animal 1 received 12 pounds of grain daily; No. 2, 7 to 8 pounds; and No. 3, 11 pounds.

In Period 2 the 10 pounds of timothy hay were displaced by 10 pounds of alfalfa hay; the corn silage remained the same; but the grain mixture was changed to one consisting of 70 parts of yellow corn, 25 parts of wheat bran, and 5 parts of oil meal. The daily amount of grain mixture allowed each animal was the same as in Period 1. This change in the proportion of ingredients in the grain mixture was made for the purpose of keeping the protein intake of the two periods much alike. The alfalfa hay used was second cutting, grown on the University's farm and *cured in the windrow for 4 days*. Although exposed for 4 days in drying it retained its green color to a considerable extent. It was fed cut as was done with the timothy hay. This period of observation was also for 4 weeks.

In Period 3 the same ration as used in Period 1 was fed, but with an addition of steamed bone meal, in amounts which would make the calcium content of the ration in Period 3 practically equal to that of the alfalfa ration or the feed mixture used in Period 2. To accomplish this the amount of steamed bone meal added daily was 200 gm. per individual. The steamed bone meal used was that prepared by the United Chemical and Organic Products Company of Chicago, and sold by them especially for animal feeding. This period of observation was for 5 weeks. We ran it for a longer time purposely in order to obliterate the effect of the possible storage, during the alfalfa period, of nutritional factors that might obscure our results on timothy hay plus steamed bone meal. It would be entirely possible that a residual effect from the alfalfa period would be of such long duration as to vitiate wholly the results of a succeeding period with timothy hay, unless run for a period sufficiently long. All the animals received distilled water and common salt throughout the entire period of observation.

In Table I the record of the calcium and phosphorus content of the feeds used is given.

In Tables II to IV inclusive are the records of income and outgo of calcium and phosphorus for the 3 animals.

In Table V the data on the total calcium and inorganic phosphorus content of the blood taken from the jugular vein are given.

These determinations were made on the serum, the inorganic phosphorus by the method of Marriott and Haessler (3) and the calcium by the method of Halverson and Bergeim (4).

TABLE I.

Calcium and Phosphorus Content of the Feeds as Fed.

Material.	CaO	P_2O_5
	per cent	*per cent*
Timothy hay..............................	0.406	0.386
Alfalfa hay...............................	2.55	0.514
Corn silage..............................	0.118	0.161
Corn (grain)..............................	0.023	0.543
Oil meal.................................	0.550	2.082
Wheat bran..............................	0.158	2.796
Bone meal...............................	48.10	35.40

DISCUSSION.

As was expected, decidedly negative calcium and phosphorus balances were obtained during the timothy hay feeding period. With two of the cows (Nos. 1 and 3) whose daily milk production was above 40 pounds, the negative calcium balances were as high as 30 gm. of CaO per day and the negative phosphorus balances about the same. In the case of Animal 2 with a daily milk production of 20 to 25 pounds, the calcium loss was not so large as with the other two animals. During this period the daily calcium intake was approximately equal to the daily calcium content of the milk produced by Animals 1 and 3. Although in this period the phosphorus intake was, approximately, three times the calcium intake, yet negative phosphorus balances resulted. This would be expected. While negative calcium balances accompanied by positive phosphorus balances have been observed by several investigators, yet with a mature animal it is difficult

TABLE II.
Record of Calcium Balance of Animal 1.

Period.	CaO in feces.	CaO in urine.	CaO in milk.	Total CaO excreted.	Total CaO intake.	Balance per week.	Balance per day.	Milk per week.
Timothy hay period.								
	gm.	gm.	gm.	gm.	gm.	gm.	gm.	lbs.
Dec. 12–19	181.05	1.92	291.16	474.13	258.57	−215.56	−30.37	327.2
" 19–26	158.38	1.23	282.08	441.69	274.64	−167.05	−23.86	330.6
" 26–Jan. 2	168.54	0.54	239.68	408.76	274.64	−134.12	−19.16	332.6
Jan. 2–9	175.06	1.31	279.36	455.73	274.64	−181.09	−25.87	327.3
Alfalfa hay period.								
Jan. 9–16	526.31	5.15	269.12	800.58	935.79	+135.21	+19.31	323.7
" 16–23	702.06	4.09	282.42	988.57	935.79	−52.78	−7.54	328.4
" 23–30	692.45	3.22	258.99	954.66	935.79	−18.87	−2.69	306.4
Jan. 30–Feb. 6	792.72	2.73	258.21	1,053.66	935.79	−117.87	−16.84	309.1
Timothy hay + bone meal period.								
Feb. 6–13	767.18	1.18	244.84	1,013.20	948.04	−65.16	−9.31	293.1
" 13–20	886.24	4.01	239.22	1,129.47	948.04	−181.43	−25.92	283.3
" 20–27	863.93	2.70	237.82	1,104.45	948.04	−156.41	−22.34	284.7
" 27–Mar. 6	809.99	2.19	219.32	1,031.50	948.04	−83.46	−11.92	271.4
Mar. 6–13	809.20	1.70	234.04	1,044.94	948.04	−96.90	−13.84	292.9

Record of Phosphorus Balance of Animal 1.

Period.	P_2O_5 in feces.	P_2O_5 in urine.	P_2O_5 in milk.	Total P_2O_5 excreted.	Total P_2O_5 intake.	Balance per week.	Balance per day.
Timothy hay period.							
	gm.	gm.	gm.	gm.	gm.	gm.	gm.
Dec. 12–19	691.74	2.93	310.47	1,005.14	729.69	−275.45	−39.35
" 19–26	696.41	3.22	300.09	999.72	751.62	−248.10	−35.44
" 26–Jan. 2	687.45	3.01	276.34	966.80	751.62	−215.18	−30.74
Jan. 2–9	695.52	2.29	309.08	1,006.89	751.62	−255.27	−36.46
Alfalfa hay period.							
Jan. 9–16	664.90	7.97	306.74	979.61	742.41	−237.20	−33.88
" 16–23	553.15	15.40	300.25	868.80	742.41	−126.39	−18.05
" 23–30	480.93	13.75	288.23	782.91	742.41	−40.50	−5.78
" 30–Feb. 6	447.40	6.58	294.70	748.68	742.41	−6.27	−0.89
Timothy hay + bone meal period.							
Feb. 6–13	927.87	9.59	268.79	1,206.25	1,247.22	+40.97	+5.85
" 13–20	1,186.14	14.83	255.94	1,456.91	1,247.22	−209.69	−29.95
" 20–27	1,052.44	6.25	254.62	1,313.31	1,247.22	−66.09	−9.44
" 27–Mar. 6	1,140.26	6.38	245.20	1,391.84	1,247.22	−144.62	−20.66
Mar. 6–13	1,179.80	5.10	268.61	1,453.51	1,247.22	−206.29	−29.49

TABLE III.
Record of Calcium Balance of Animal 2.

Period.	CaO in feces.	CaO in urine.	CaO in milk.	Total CaO excreted	Total CaO intake.	Balance per week.	Balance per day.	Milk per week.
				Timothy hay period.				
	gm.	gm.	gm.	gm.	gm.	gm.	gm.	lbs.
Dec. 12–19	174 35	0 95	141 90	317 20	241.28	−75.92	−10.84	164.5
" 19–26	189.34	0.74	144 17	334 25	257.35	−76.90	−10.98	158.8
" 26–Jan. 2	167 36	1.94	149.94	319.24	237 58	−81.66	−11.66	166.8
Jan. 2–9	219.69	1 02	135.11	355.82	234.28	−121.54	−17.36	155.0
				Alfalfa hay period.				
Jan. 9–16	604.36	0.80	150 33	755.49	903.85	+148.36	+21.19	153.3
" 16–23	780.89	2.22	164 09	947.20	903.85	−43.35	−6.19	165.8
" 23–30	762.77	2.39	152.05	917.21	903.85	−13.36	−1.91	156.5
" 30–Feb. 6	754.18	1.51	159.34	915.03	903.85	−11.18	−1.59	164.0
				Timothy hay + bone meal period.				
Feb. 6–13	877.90	2.50	141 64	1,022 02	907 68	−114 34	−16.33	143.1
" 13–20	791.74	1.53	133 62	926.89	907 68	−19 21	−2.74	139.5
" 20–27	834.15	2.22	119 80	956.17	907 68	−48.49	−6.93	128.2
" 27–Mar. 6	670.03	1.57	104 49	776 09	907.68*	+131 59	+18.79	109.5
Mar. 6–13	879.93	1 66	107.06	988 65	907 68	−80 97	−11.56	113.3

Record of Phosphorus Balance of Animal 2.

Period.	P_2O_5 in feces.	P_2O_5 in urine.	P_2O_5 in milk.	Total P_2O_5 excreted.	Total P_2O_5 intake.	Balance per week.	Balance per day.
				Timothy hay period.			
	gm.	gm.	gm.	gm.	gm.	gm.	gm.
Dec. 12–19	621.64	3.63	164 30	789 57	568.57	−221.00	−31.57
" 19–26	583 85	3 97	166 52	754 34	590 50	−163.84	−23.40
" 26–Jan. 2	502.07	3 64	168 11	673 82	532.15	−141.67	−20.28
Jan. 2–9	701.10	4 77	147.07	852.94	522.43	−330.51	−47.21
				Alfalfa hay period.			
Jan. 9–16	451.23	4 52	163.56	619.31	528.85	−90.46	−12.92
" 16–23	413 74	2.63	173 88	590 25	528.85	−61.40	−8.77
" 23–30	391 97	7 77	161.99	561.73	528.85	−32.88	−4.69
" 30–Feb. 6	390.45	3 82	170 50	564.77	528.85	−35.92	−5.13
				Timothy hay + bone meal period.			
Feb. 6–13	850 72	5 28	144.87	1,000.87	1,018.03	+17.16	+2.45
" 13–20	1,015 45	4 01	142.61	1,162.07	1,018.03	−144.04	−20.58
" 20–27	1,115.16	4.59	125 62	1,245 37	1,018.03	−227.34	−32.47
" 27–Mar. 6	840.39	4 13	115 54	960 06	1,018.03	+57.97	+8.28
Mar. 6–13	1,048.24	4 93	122.28	1,175.45	1,018.03	−157.42	−22.49

* Indicated positive balance not an actuality, due to incomplete consumption not here recorded.

TABLE IV.
Record of Calcium Balance of Animal 3.

Period.	CaO in feces.	CaO in urine.	CaO in milk.	Total CaO excreted.	Total CaO intake.	Balance per week.	Balance per day.	Milk per week.
Timothy hay period.								
	gm.	gm.	gm.	gm.	gm.	gm.	gm.	lbs.
Dec. 12–19	259.31	0.44	227.22	486.97	254.25	−232.72	−33.24	272.4
" 19–26	246.47	0.38	231.43	478.28	270.32	−207.96	−29.71	283.2
" 26–Jan. 2	246.94	0.69	241.24	488.87	270.32	−218.55	−31.22	273.9
Jan. 2–9	258.21	0.56	212.29	471.06	270.32	−200.74	−28.67	260.7
Alfalfa hay period.								
Jan. 9–16	458.49	0.89	207.49	666.87	866.50	+199.63	+28.52	253.9
" 16–23	753.58	1.69	207.39	962.66	932.16	−30.50	−4.36	251.0
" 23–30	736.40	0.89	207.32	944.61	932.16	−12.45	−1.78	251.7
" 30–Feb. 6	750.37	2.41	205.83	958.61	932.16	−26.45	−3.78	248.1
Timothy hay + bone meal period.								
Feb. 6–13	955.96	2.40	198.93	1,157.29	943.72	−213.57	−30.51	235.6
" 13–20	887.04	2.36	184.05	1,073.45	943.72	−129.73	−18.53	233.0
" 20–27	800.86	2.09	181.91	984.86	943.72	−41.14	−5.88	222.6
" 27–Mar. 6	774.24	1.67	186.06	961.97	943.72	−18.25	−2.61	215.7
Mar. 6–13	877.89	1.87	183.76	1,063.52	943.72	−119.80	−17.11	215.3

Record of Phosphorus Balance of Animal 3.

Period.	P_2O_5 in feces.	P_2O_5 in urine.	P_2O_5 in milk.	Total P_2O_5 excreted.	Total P_2O_5 intake.	Balance per week.	Balance per day.
Timothy hay period.							
	gm.	gm.	gm.	gm.	gm.	gm.	gm.
Dec. 12–19	695.48	1.97	279.08	976.53	696.04	−280.49	−40.07
" 19–26	684.27	4.01	280.28	968.56	717.97	−250.59	−35.79
" 26–Jan. 2	718.85	2.24	279.79	1,000.88	717.97	−282.91	−40.41
Jan. 2–9	704.90	2.23	257.61	964.74	717.97	−246.77	−35.25
Alfalfa hay period.							
Jan. 9–16	499.43	3.36	266.28	769.07	654.47	−114.60	−16.37
" 16–23	459.61	2.19	247.28	709.08	704.81	−4.27	−0.61
" 23–30	460.25	4.24	237.27	701.76	704.81	+3.05	+0.43
" 30–Feb. 6	443.04	2.87	249.93	695.84	704.81	+8.97	+1.26
Timothy hay + bone meal period.							
Feb. 6–13	1,282.08	5.23	236.39	1,523.70	1,213.57	−310.13	−44.30
" 13–20	1,308.03	4.90	235.89	1,548.82	1,213.57	−335.25	−47.89
" 20–27	1,166.98	5.60	218.29	1,390.87	1,213.57	−177.30	−25.33
" 27–Mar. 6	1,101.97	3.74	216.42	1,322.13	1,213.57	−108.56	−15.51
Mar. 6–13	1,050.80	5.00	223.83	1,279.63	1,213.57	−66.06	−9.44

to conceive how such a situation could prevail for any length of time with calcium phosphate as the main compound in which these two elements are stored. In spite of negative calcium and phosphorus balances the milk production was well sustained in this period by all the animals.

We changed the animals suddenly to the alfalfa hay ration. From our previous results (1) we expected positive calcium and phosphorus balances to prevail. We did have a positive calcium balance with all animals for the 1st week, but after that negative

TABLE V.

Calcium and Inorganic Phosphorus in the Blood at Different Stages of the Experiment.

Animal No.	Inorganic P per 100 cc. of serum.	Ca per 100 cc. of serum.	Time of taking sample.
	mg.	*mg.*	
1	2.25	22 01	End of timothy hay period.
2	2.63	22.60	
3	2.83	24.42	
1		16.34	4 days after change to alfalfa hay.
2		15.65	
3		15.90	
1	5 97	8.79	End of alfalfa hay period.
2	3.83	9.81	
3	2.90	9.91	
1	5.75	16.10	End of timothy hay plus steamed
2	4.38	16.75	bone meal period.
3	4.90	16.75	

balances resulted. The positive calcium balance in the 1st week was due to the high intake of this element incident to the change in the ration and to the lag in elimination, by way of the feces, of the residues from the first few days of alfalfa hay feeding. The true situation established in the 2nd week was a slight but distinct negative calcium balance for all the animals in the alfalfa hay period. The phosphorus balances were also negative for Animals 1 and 2 during the entire period, but both negative and positive—or equilibrium—for Animal 3. As noted above, these

results differ from those secured by us in an earlier experiment where the alfalfa hay had been cured under caps and out of long exposure to sunlight. The question as to what is the actual difference between alfalfa hay cured under caps and alfalfa hay cured in the windrow with a comparatively distinct difference in respect to the time of exposure to light and air cannot be answered at present.

It is known that the fat-soluble vitamine is destroyed by long exposure to light and air. For example, when melted butter fat has been poured into shallow dishes, thereby presenting a large surface, and then exposed to direct light and bleached, the fat-soluble vitamine is destroyed. We are not assuming that the fat-soluble vitamine and the vitamine of green plant tissue influencing calcium assimilation are identical and consequently may be unjustified in predicting parallel behavior. In the absence of full experimental data on the effect of aging, bleaching, and exposure of plant tissue to light upon the destruction of this unknown factor affecting calcium assimilation, discussion of the subject is useless. We have, however, shown (5) that fresh green oats as compared with oat straw is much more effective in increasing calcium assimilation. The oat straw is also low in the fat-soluble vitamine. On the other hand, oat hay dried out of direct sunlight but in a fairly well lighted attic appeared to retain the properties of the fresh green oats.

The fact that with this alfalfa hay positive calcium balances could not be obtained places these data in agreement with those secured by Forbes (2) and by Meigs and his associates (6). Our earlier data, contrasted with these later results, showed that it was possible to provide a ration of natural and unsupplemented materials which could maintain calcium and phosphorus equilibrium in high milking animals. Just what happened in the treatment of these food materials, particularly the roughages, to bring about these differences in behavior must be a subject for further inquiry.

It should be emphasized that the degree of negative calcium and phosphorus balances observed with the alfalfa hay in contrast with the timothy hay is not large and could no doubt be maintained for a very long time without serious results to the animal.

Fortifying timothy hay with bone meal did not result in main-

taining positive calcium and phosphorus balances. The losses
of these elements, particularly calcium, were reduced as compared
with the period on unsupplemented timothy hay, but were not
changed to a condition of storage or equilibrium. The combina-
tion of timothy hay and bone meal was not as effective as the
alfalfa hay alone. We interpret these results as indicating that
the timothy hay possessed less of the vitamine assisting calcium
assimilation than did the alfalfa hay. The question of the solu-
bility of the combination of calcium administered is often raised,
but we believe that question to be only of secondary importance
—the primary factor being the supply in the ration of the organic
factor influencing calcium assimilation. For example, in experi-
ments with swine we have seen the development of rickets meas-
urably delayed by the use of calcium acetate as compared with
the more insoluble calcium phosphate (floats), but the end-re-
sult was the same. Forbes (2) likewise found with milking cows
negative calcium balances where fortification of the ration was
made either with calcium lactate, calcium chloride, or bone flour.

At regular intervals during the course of these experiments
blood was taken from the jugular vein for inorganic phosphorus
and total calcium determinations. The time of taking the
blood in reference to the ration fed is given in Table V. Our
data on the inorganic phosphorus content of the blood are diffi-
cult of interpretation. During the timothy hay period the amount
of inorganic phosphorus in the blood of all these animals was low
as compared with data secured in the later periods of higher cal-
cium intake and also with data secured by Meigs and his associates
(6) on cows receiving a ration of grains, corn silage, and alfalfa
hay. The data are more in harmony with the condition observed
by Howland and Kramer (7) in rachitic children where the inor-
ganic phosphorus content of the blood is unusually low in amount.
Although decided but reduced negative phosphorus balances
prevailed in both the alfalfa hay period and the period of timothy
hay plus bone meal, yet the inorganic phosphorus content of the
blood was in all cases but one, distinctly higher than in the tim-
othy hay period.

The results on total calcium in the blood are exceedingly in-
teresting. From the recent literature on the subject the inference
is gained that the calcium content of the blood is subject to but

slight variations—that the range may be from 9 to 12 mg. per 100 cc. of plasma or serum. This has been the amount found in the plasma of normal human beings and of horses, dogs, and cattle (6). That variations from the above figures may occur is also evident from a limited number of observations recorded in the literature. By feeding calcium lactate to two dogs Boggs (8) found an increase of 36 per cent in the calcium content of the whole blood or an increase from 6.3 mg. per 100 cc. of blood to 8.6 mg. Allers and Bondi (9) doubled the calcium content of the blood of rabbits by feeding large doses of hydrochloric acid. Meigs (6) calls attention to the correlation between the work of Hasselbalch and Gammeltoft (10) who showed that in pregnancy in human beings there is a definite tendency for the H ion concentration of the blood at a fixed CO_2 tension to be increased and the results of Lamers (11) who found an increased plasma calcium in pregnancy and particularly during labor. Meigs found the plasma calcium in new-born heifers as high as 13.7 mg. per 100 cc., while the amount in the plasma of pregnant and lactating cows was approximately 10 mg.

This tendency of the calcium content of the blood to variation led Meigs and his associates to adopt as an explanation of this phenomenon the modern view-point of the influence of reaction—H ion concentration—on the bicarbonate content of the blood and the inverse relation of the latter to the calcium concentration in the blood. In other words, a higher acidity of the blood means a decreased bicarbonate concentration with a resultant possibility of a higher concentration of calcium. This appears to be a logical deduction and explanation for such variations.

Unfortunately, we made no bicarbonate determinations in the blood of our animals, and consequently cannot present data bearing on the above theory. A comparatively low intake of calcium, such as prevailed during the timothy hay period, did not result in a low calcium concentration of the blood plasma; but, on the contrary, an unusually high calcium concentration prevailed. In a recent publication (12) we recorded a very limited amount of data secured with *dry* cows which showed that the alkaline reserve of the blood in the case of cows receiving a ration low in calcium content—oat grain plus oat straw—as com-

pared with a calcium-rich ration—alfalfa hay—was not different. But in the case of heavy milking cows the situation may be entirely different. If the above theory, explaining calcium variations in the blood, is correct then the alkaline reserve of the bloods of these animals during the timothy hay period must have been reduced.

With the change to the alfalfa ration the calcium concentration of the blood decreased from over 20 mg. per 100 cc. of serum on the timothy hay to approximately 10 mg. per 100 cc. of serum. This change was a gradual one as shown by the fact that 4 days after the change to the alfalfa hay the calcium concentration of the blood had decreased to about 16 mg. per 100 cc. of serum. With the return to the timothy hay plus bone meal ration the blood calcium again rose from approximately 10 to 16 mg. per 100 cc. of serum, but not to as high a level as when the timothy hay alone was fed. As shown in the table of calcium balances some use, but not a complete one, of the bone meal had been made.

Before permanently incorporating into this paper the data on the calcium content of the blood of these animals we submitted them for criticism to Dr. E. B. Meigs, of the Dairy Division, United States Department of Agriculture. We did this because of the unusual variations observed. It so happend that Dr. Meigs had accumulated data on the same problem in the feeding of milking cows with results in reference to blood calcium directly contrary to our own. In fact, on the timothy hay he used as contrasted with alfalfa hay the plasma calciun was reported somewhat lower. Fortunately, we had a group of high milking cows that had been for several months on the same timothy hay, plus corn silage and a grain mixture, as used in our metabolism experiments and another group of cows upon alfalfa hay, corn silage, and a grain mixture. This alfalfa hay was not the same as that used with the three cows involved in the balance experiments. These two groups of cows were not in the metabolism stalls, but being managed as in ordinary herd management. In addition to the rations mentioned they had received daily per individual $\frac{1}{2}$ pound of steamed bone meal for approximately 60 days during the dry period just before parturition. They had been milking about 3 months when the blood samples were taken.

Their daily milk production was from 35 to 50 pounds of milk per individual.

Blood was taken from the jugular vein and analyses for total calcium were made both by the Halverson method, which we had always used in our work, and by the method Meigs had used

TABLE VI.

Calcium by Two Methods in the Blood of a Second Group of Cows Receiving Low and High Calcium Intake.

Cow No.	Determi-nation.	Halverson method. Mg. per 100 cc. of plasma.	Determi-nation.	Meigs' method. Mg. per 100 cc. of plasma.	Remarks.
1	a............	12 26	a............	8.99	Timothy hay.
	b............	12 34	b............	10.70	
	Average .	12.30	Average .	9 85	
2	a............	11.96	a............	9.33	Timothy hay.
	b............	11.64	b............	9.58	
	Average .	11.80	Average .	9.45	
4	a............	10.58	a............	8 63	Timothy hay.
	b............	10.35	b............	9.08	
	Average .	10.46	Average .	8.85	
5	a............	11.96	a............	10 02	Alfalfa hay.
	b............	12.26	b............	9.65	
	Average .	12.11	Average .	9.83	
6	a............	11.11	a............	9.31	Alfalfa hay.
	b............	Lost.	b............	9.26	
	Average .	11.11	Average .	9.29	
7	a............	10.03	a............	9 29	Alfalfa hay.
	b............	9.81	b............	9.34	
	Average .	9.92	Average .	9.32	
8	a............	10.58	a............	8 71	Alfalfa hay.
	b............	10.35	b............	7.45	
	Average .	10.47	Average .	8.08	

(6). We believe that the physiological status of these animals could not have been different from the three involved in the complete balance experiments whose blood calcium varied greatly. However, in this opinion we may be wrong because of the longer

exposure of these animals to the particular ration. The data on the blood analysis for calcium of the two groups of cows and by the two different methods are given in Table VI.

A survey of the data shows that the two methods varied by about 20 per cent, the Halverson method consistently running higher. More important than the comparison of the methods was the uniform amount of calcium in the blood of these animals. Those receiving the mineral-poor ration—timothy hay—had approximately as much calcium in the blood as those receiving the alfalfa hay. These data are very different from the blood calcium records for the animals in the metabolism experiments as shown in Table V. We do not believe that the difference is one of analytical error as the work was done using the same reagents and by the same analyst. Whether distinct differences in rations in respect to mineral content and with but limited time for adjustment such as prevailed with the metabolism cows can explain these differences is a problem. At present we have no suggestion to offer as an explanation of the apparent discrepancy.

SUMMARY.

1. Cows producing from 20 to 45 pounds of milk per day were in negative calcium and phosphorus balances on rations composed of grains and their by-products, corn silage and timothy hay.

2. Substituting alfalfa hay for the timothy hay reduced the losses but did not bring about calcium and phosphorus equilibrium; negative balances continued. These data with this alfalfa hay are distinctly contrary to our earlier observations where positive calcium balances were obtained with an alfalfa hay (1). The alfalfa hay used in the earlier experiments was cured under caps; while the alfalfa hay used in this later experiment was cured in the windrow with exposure to air and light for 4 days. These differences in effect of the two alfalfa hays may be attributed to a difference in the degree of destruction during the curing process of the vitamine assisting calcium assimilation.

3. Supplementing the timothy hay with bone meal did not result in establishing positive calcium and phosphorus balances or even equilibrium; although the losses of these elements were reduced as compared with the unsupplemented timothy hay.

4. Blood calcium was comparatively high in amount during the timothy hay period when the calcium losses were greatest. It was between 20 and 25 mg. per 100 cc. of serum. In the alfalfa period it dropped to approximately 10 mg. per 100 cc. of serum, but rose again to 16 mg. during the timothy hay + bone meal period. With two other groups of cows not in metabolism stalls, but one group receiving timothy hay, or a low calcium intake, and the other group receiving an alfalfa hay, or a liberal calcium intake, no such difference in the blood calcium was observed.

5. Inorganic phosphorus in the blood was low in amount during the timothy hay period, but considerably higher in the alfalfa and timothy hay plus bone meal periods.

BIBLIOGRAPHY.

1. Hart, E. B., Steenbock, H., Hoppert, C. A., and Humphrey, G. C., *J. Biol. Chem.*, 1922, liii, 21.
2. Forbes, E. B., Beegle, F. M., Fritz, C. M., Morgan, L. E., and Rhue, S. N., *Ohio Agric. Exp. Station, Bull. 295*, 1916; *Bull. 308*, 1917. Forbes, E.B., Halverson, J. O., and Morgan, L. E., *Ohio Agric. Exp. Station, Bull. 330*, 1918.
3. Marriott, W. McK., and Haessler, F. H., *J. Biol. Chem.*, 1917, xxxii, 241.
4. Halverson, J. O., and Bergeim, O., *J. Biol. Chem.*, 1917, xxxii, 159.
5. Steenbock, H., and Hart, E. B., *J. Biol. Chem.*, 1913, xiv, 59. Hart, E. B., Steenbock, H., and Hoppert, C. A., *J. Biol. Chem.*, 1921, xlviii, 33.
6. Meigs, E. B., Blatherwick, N. R., and Cary, C. A., *J. Biol. Chem.*, 1919, xl, 469.
7. Howland, J., and Kramer, B., *Am. J. Dis. Child.*, 1921, xxii, 105.
8. Boggs, T. R., *Bull. Johns Hopkins Hosp.*, 1908, xix, 201.
9. Allers, R. A., and Bondi, S., *Biochem. Z.*, 1907, vi, 366.
10. Hasselbalch, K. A., and Gammeltoft, S. A., *Biochem. Z.*, 1915, lxviii, 206.
11. Lamers, A. J. M., *Z. Geburtsh. u. Gynäk.*, 1912, lxxi, 393.
12. Hart, E. B., Steenbock, H., and Humphrey, G. C., *Wisconsin Exp. Station, Research Bull. 49*, 1920.

UNSATURATED FATTY ACIDS OF BRAIN CEPHALINS.

By P. A. LEVENE and IDA P. ROLF.

Cephalin was recognized as a substance distinct from lecithin by Thudichum.[1] According to this author, the differences in the properties of the two substances were due to the differences in the structure of the fatty acids and of the bases which enter into their respective molecules. As regards the structure of the base, the views of Thudichum were confirmed by the more recent and more modern work. His views on the nature of the fatty acids also, seemed to have been corroborated by later work of Cousin,[2] Falk,[3] and especially that of Parnas.[4]

Recent work on the fatty acids of lecithin has led to the discovery that at least two and probably three unsaturated and two saturated acids are present in the mixed lecithins.[5,6,7] In the light of this experience it seemed desirable to subject the problem of the nature of the fatty acids of cephalin to a new investigation.

The material used for the present work was entirely free from lecithin and neutral fats. It was also entirely free from substances containing non-amino nitrogen. As is seen from its elementary composition this material consisted in part of cephalin which had undergone partial hydrolysis. It was as pure as the best that had been used by the previous workers. From this material pure cephalin undoubtedly can be prepared. The purification is

[1] Thudichum, J. L. W., A treatise on the chemical constitution of the brain, London, 1884.

[2] Cousin, M. H., *Compt. rend. Soc. biol.*, 1906, lxi, 23.

[3] Falk, F., *Biochem. Z.*, 1909, xvi, 187.

[4] Parnas, J., *Biochem. Z.*, 1909, xxii, 411; 1913, lvi, 17.

[5] Levene, P. A., and Simms, H. S., *J. Biol. Chem.*, 1922, li, 285.

[6] Levene, P. A., and Rolf, I. P., *J. Biol. Chem.*, 1922, li, 507.

[7] Levene, P. A., and Rolf, I. P., *J. Biol. Chem.*, 1921, xlvi, 363.

time-consuming and results in much loss of material. In the course of the work the highly unsaturated acids may undergo oxidation. Owing to these considerations the purification of the material was limited to the stage in which it was free from non-amino nitrogen, in other words, free from lecithin. Such material may yield fewer, not a greater number of fatty acids than are present in the pure cephalin.

Following the course of analysis as employed in the work on lecithin, it was possible to isolate from cephalin two unsaturated fatty acids; namely, oleic and arachidonic acid. The oleic acid was separated first as a barium salt (mixed with the saturated acids). This was then converted into the free acid and as such it was identified by the iodine number and by the fact that on hydrogenation it yielded stearic acid. The presence of arachidonic acid was demonstrated by the isolation of its octabromide, and by the isolation of arachidic acid from the product of hydrogenation of the unsaturated fatty acids.

Since a minor fraction only, of the highly unsaturated acids was converted into the insoluble octabromides, there still remains the possibility of the discovery of other unsaturated acids. At present no evidence is available in support of the assumption that linolic acid is the principal unsaturated acid of cephalin.

From the saturated fatty acid fraction, stearic acid was isolated in pure condition. This fraction, however, also contained other acids of a different composition. This material is now under further investigation.

<div align="center">EXPERIMENTAL.</div>

<div align="center">A. Preparation of Cephalin.</div>

Ox brains were extracted first with acetone and subsequently with ether containing 5 per cent of water. From the concentrated ethereal extract, the bulk of the cerebrosides was removed by filtration at 0°C. The filtrate was then fractionated with alcohol. The precipitate after purification yielded material which contained all of its nitrogen as amino nitrogen. Two methods of purification were employed on each sample. The first is that described by Levene and Ingvaldsen.[8] The crude cephalin is

[8] Levene, P. A., and Ingvaldsen, T., *J. Biol. Chem.*, 1920, xliii, 359.

dissolved in glacial acetic acid. This solution is allowed to stand for 18 hours at 15°C. The precipitate, consisting of cholesterol and cerebrosides, is removed by filtration. To the filtrate about 10 volumes of alcohol are added and again the mixture is allowed to stand over night at 0°C. and the resultant precipitate removed. The filtrate, after concentration *in vacuo*, is emulsified with water and precipitated with acetone. This method of purification yields material with a markedly higher carbon content than that of the original mixture.

The second method of purification permits the separation of two types of material, one, the amino content of which is greatly increased, and one of a greater carbon content than the original material. Crude cephalin is dissolved in a minimal quantity of warm ether. To this, warm alcohol is gradually added, not, however, in sufficient quantity to cause precipitation. A saturated alcoholic solution of cadmium chloride is added, until no further precipitation occurs. The mother liquor (A) is decanted from the precipitate (B) and concentrated to dryness under diminished pressure. The residue is extracted with ether and the ethereal solution, after filtration from the inorganic precipitate, is again concentrated, emulsified with water, and precipitated with acetone. The yields of such precipitates have been as high as 20 per cent of the starting material. Since this consists only of the more highly unsaturated fraction, great variation is experienced between different lots of cephalin. The precipitate (B) referred to above, contains lecithin cadmium chloride (C) and "cephalin" (D) of a lower carbon content. The latter is separated by its solubility in glacial acetic acid. This procedure constitutes the simplest means thus far used for the preparation of "cephalin" of high amino content.

Of such fractionation the following results are typical:

No. 216 was the alcohol-soluble fraction of an ethereal extract. It was purified by the glacial acetic acid-alcohol method, and analyzed as follows:

0.1060 gm. substance: 0.0926 gm. H_2O, 0.2426 gm. CO_2, and 0.0126 gm. ash.

0.1880 "   "   required (Kjeldahl) 2.85 cc. 0.1 N acid.

0.2819 "   "   : (fusion) 0.0412 gm. $Mg_2P_2O_7$.

0.2 gm. was dissolved in glacial acetic acid.

5 cc. of this solution required (Kjeldahl) 1.25 cc. 0.1 N acid.

2 " " " " : (Van Slyke) 0.71 cc. N_2 at 24°C., 766.5 mm.

0.2781 gm. substance absorbed 0.2154 gm. iodine.

Found (No. 216). C 64.10, H 9.92, N 2.02, P 4.07.

$$\frac{\text{Amino } N_2}{\text{Total } N_2} = \frac{56}{100} \qquad \text{Iodine No.} = 80$$

No. 216 was fractionated through the use of cadmium chloride, as described above. The material obtained from the mother liquor (A) analyzed as follows:

No. 220. 0.1090 gm. substance: 0.0984 gm. H_2O, 0.2574 gm. CO_2, and 0.0112 gm. ash.

0.1838 gm. substance required (Kjeldahl) 2.65 cc. of 0.1 N acid.

0.2757 " " : (fusion) 0.0400 gm. $Mg_2P_2O_7$.

0.2 gm. substance was dissolved in glacial acetic acid.

5 cc. of this solution required (Kjeldahl) 0.75 cc. 0.1 N acid.

2 " " " " : (Van Slyke) 0.55 cc. of N_2 at 24°C., 764.5 mm.

0.2724 gm. substance absorbed 0.2630 gm. iodine.

Found (No. 220). C 65.05, H 10.23, N 2.01, P 4.04.

$$\frac{\text{Amino } N_2}{\text{Total } N_2} = \frac{73}{100} \qquad \text{Iodine No.} = 96$$

The lecithin cadmium chloride salt (C), insoluble in glacial acetic acid, contained 25 per cent amino nitrogen and had an iodine number of 40.

That fraction (D) which was extracted by the glacial acetic acid had the following composition:

0.1040 gm. substance: 0.0884 gm. H_2O, 0.2306 gm. CO_2, and 0.0144 gm. ash.

0.1867 gm. substance required (Kjeldahl) 2.70 cc. 0.1 N acid.

0.2801 " " : (fusion) 0.0410 gm. $Mg_2P_2O_7$.

0.2 gm. substance was dissolved in glacial acetic acid.

5 cc. of this solution required (Kjeldahl) 0.75 cc. of 0.1 N acid.

2 " " " " : (Van Slyke) 1.10 cc. N_2 at 22°C., 773.2 mm.

0.2915 gm. substance absorbed 0.2249 gm. iodine.

Found (No. 218). C 62.11, H 9.90, N 2.02, P 4.07.

$$\frac{\text{Amino } N_2}{\text{Total } N_2} = \frac{103}{100} \qquad \text{Iodine No.} = 77$$

This material served as the source of the fatty acids to be described later. The cephalin was boiled for 12 hours with 10

per cent hydrochloric acid, and the fatty acids were ex
with ether. Owing to the presence of considerable re
material which was not wholly removed even by this metho
was found expedient to esterify the mixed acids and extract the
esters with ether. This solution was thoroughly washed with a
solution of dilute sodium carbonate, and repeatedly filtered.
The esters were saponified with barium hydroxide and the barium
salts separated into two fractions by the method described in a
previous publication.[6]

B. Saturated Acids.

From the barium salts which were insoluble in benzene-alcohol,
the acids were liberated. These acids, consisting of mixed satu-
rated and oleic acids, were converted into lead salts and the
lead oleate was extracted by a large volume of ether. The residual
salts of the saturated acids were dissolved in toluene and from
them the free acids were liberated by hydrogen sulfide. They
were then esterified and the methyl esters after recrystallization
were fractionated by distillation at a pressure of 1.9 mm.

The esters were saponified, and analyses, melting points, and
molecular weight determinations made on the free acids. The
drying, analyses, and molecular weight determinations were
carried out in the manner described in previous papers.[6,7] The
melting points, however, were taken in a bath which was con-
tinuously stirred. A corrected thermometer was used, and the
time interval per degree rise in temperature was 6 seconds.

No. 572. 0.1006 gm. substance: 0.1166 gm. H_2O and 0.2788 gm. CO_2.
1.0056 "   "   required for neutralization 7.05 cc. of
0.5 N NaOH, corresponding to a mol. wt. of 285.

No. 562. 0.0993 gm. substance: 0.1186 gm. H_2O and 0.2766 gm. CO_2.
1.1524 "   "   required for neutralization 7.96 cc. of
0.5 N NaOH.

No. 563. 0.1010 gm. substance: 0.1162 gm. H_2O and 0.2724 gm. CO_2.
1.0378 "   "   required for neutralization 7.10 cc. of
0.5 N NaOH.

No. 564. The residue, was insufficient for either molecular weight or
analysis.

$C_{18}H_{36}O_2$. Calculated. C 75.98, H 12.76 (Mol. wt. = 284. Melting
point = 70-71°C.)

	No.	Boiling point of ester. Pressure 1.9 mm.	Weight of ester.	Analysis of acid.		Molecular weight of acid.	Melting point of acid.
				C	H		
		°C.	gm.				°C.
Found.	572	165–172	5	75.57	12.97	285	70–71
"	562	172	12	75.96	13.36	289	64–65
"	563	175–185	5	74.03	12.87	292	66–67
"	564	Residue.	1				76

C. Oleic Acid.

The lead salt which was extracted by ether from the saturated lead salts, was converted into the free acid. This was a light yellow mobile liquid. 0.2540 gm. of substance absorbed 0.2273 gm. of iodine, corresponding to an iodine number of 90. The iodine number of oleic acid is 90.

This substance was hydrogenated and the saturated acid had the following analysis, molecular weight, and melting point:

0.1008 gm. substance: 0.1170 gm. H_2O and 0.2800 gm. CO_2.
0.9492 "   "   required for neutralization 6.67 cc. of 0.5 N NaOH.
It melted at 70–71°C.
$C_{18}H_{36}O_2$.   Calculated.   C 75.98, H 12.76. (Mol. wt. = 284.)
                                         Melting point = 70–71°C.)
             Found (No. 571).   C 75.75, H 12.99. (Mol. wt. = 284.)
                                         Melting point = 70–71°C.)

D. The More Unsaturated Acids.

The barium salts which were soluble in the benzene-alcohol mixture were converted into free acids. As such they formed a dark mobile liquid having an iodine number of 140.

0.2611 gm. of substance absorbed 0.3677 gm. of iodine. 60 gm. of No. 517 were esterified and the ethyl esters hydrogenated by Paal's method. The unsaturated esters were recrystallized from alcohol, and fractionated by distillation at 1.9 mm. pressure. After saponification, the acids gave the analyses, molecular weights, and melting points recorded below.

No. 549. (Mixed esters before fractionation after saponification to the acid.) 0.1008 gm. substance: 0.1062 gm. H_2O and 0.2642 gm. CO_2.
No. 550. 0.0978 gm. substance: 0.0772 gm. H_2O and 0.2080 gm. CO_2.
         0.9637 "   "   required for neutralization 4.70 cc.
                                            0.5 N NaOH.

No. 551. 0.1010 gm. substance: 0.1154 gm. H_2O and 0.2562 gm. CO_2.
          1.0130 "   "   required for neutralization 5.85 cc. of
                                                              0.5 N NaOH.

No. 552. 0.1008 gm. substance: 0.1156 gm. H_2O and 0.2828 gm. CO_2.
          1.0721 "   "   required for neutralization 5.73 cc. of
                                                              0.5 N NaOH.

No. 553. 0.1002 gm. substance: 0.1146 gm. H_2O and 0.2830 gm. CO_2.
          0.9228 "   "   required for neutralization 6.12 cc. of
                                                              0.5 N NaOH.

No. 554. 0.1009 gm. substance: 0.1190 gm. H_2O and 0.2838 gm. CO_2.
          1.0647 "   "   required for neutralization 6.25 cc. of
                                                              0.5 N NaOH.

No. 555. 0.1010 gm. substance: 0.1204 gm. H_2O and 0.2854 gm. CO_2.
          1.0727 "   "   required for neutralization 6.00 cc. of
                                                              0.5 N NaOH.

No. 556. 0.1008 gm. substance: 0.1214 gm. H_2O and 0.2862 gm. CO_2.
          1.0645 "   "   required for neutralization 5.95 cc. of
                                                              0.5 N NaOH.

Nos. 555 and 556 were combined and purified by conversion
into the lead salt. The liberated acids were fractionally distilled
and the lower fraction (No. 573) gave the following analysis and
neutralizaton value.

0.0865 gm. substance: 0.1024 gm. H_2O and 0.2432 gm. CO_2.
0.3626 "   "   required for neutralization 2.30 cc. of 0.5 N NaOH.
$C_{18}H_{36}O_2$. Calculated. C 75.98, H 12.76. (Mol. wt. = 284. Melting
                                                            point = 70–71°C.)
$C_{20}H_{40}O_2$. Calculated. C 76.95, H 12.81. (Mol. wt. = 312. Melting
                                                            point = 75–77°C.)

	No.	Boiling point of ester. Pressure 1.9 mm.	Weight of ester.	Analysis of acid.		Molecular weight of acid.	Melting point of acid.
				C	H		
		°C.	gm.				°C.
Found.	549			71.47	11.79		
	(Mixed esters.)						
"	550	170–185	3	57.99	8.83	410	
"	551	185–190	8	69.17	12.78	347	62–63
"	552	185–195	6	76.51	12.83	373	58–59
"	553	190–207	8	77.04	12.80	316	62–63
"	554	195–205	5	76.70	13.19	341	66–67
"	555	210–220	4	77.05	13.33	357	72
"	556	220–230	2.5	77.42	13.47	374	72
"	573			76.67	13.29	315	76

E. Bromine Addition Products of the More Highly Unsaturated
Acids.

50 gm. of No. 217 were brominated by the method described in a previous paper.[6] The precipitated bromide, after repeated extraction with ether, weighed 15 gm., and gave the following analysis:

No. 521. 0.1062 gm. substance: 0.0348 gm. H_2O and 0.0984 gm. CO_2.
          0.2000 "   "   : (Carius) 0.3158 gm. AgBr.
          $C_{20}H_{32}O_2Br_8$. Calculated.   C 25.43, H 3.42.
                          Found (No. 521).   " 25.26, " 3.66.

This material, after recrystallization from a very large volume of toluene was a white amorphous powder, which darkened slightly when heated above 225°C. and sintered at 250°C. Further heating charred without melting it.

Repeated attempts were made to isolate linolic tetrabromide from the bromination mother liquor, in this, and several similar experiments. All of these efforts were, however, unsuccessful.

A large amount of dark, semiliquid material was recovered after evaporation of the glacial acetic acid. From it, on addition of gasoline, a small amount of granular substance was deposited, consisting of impure octabromide, as indicated by its analysis.

0.1036 gm. substance: 0.0354 gm. H_2O and 0.1056 gm. CO_2.
0.2022 "   "   : (Carius) 0.3046 gm. AgBr.
              Found (No. 507). C 27.79, H 3.82, Br 64.11.

The mother liquor was concentrated to dryness *in vacuo*, and the residue dissolved in absolute methyl alcohol. Powdered zinc was added, the solution heated, and hydrogen chloride passed through until a vigorous reaction was established. After 3 hours, the reaction liquor was diluted with water, and the precipitated oil extracted with ether. The residue, after evaporation of the solvent was dissolved in acetic acid, and again brominated. In this way, an additional 5 gm. of octabromide were separated, but no tetrabromide could be isolated. This bromide analyzed as follows:

0.1042 gm. substance: 0.0390 gm. H_2O and 0.0962 gm. CO_2.
0.2030 "   "   : (Carius) 0.3216 gm. AgBr.
              Found (No. 541). C 25.17, H 4.18, Br 67.42.

UNSATURATED FATTY ACIDS OF BRAIN LECITHINS.

By P. A. LEVENE and IDA P. ROLF.

In previous publications from this laboratory it was shown that the mixed lecithins from the liver[1] and from the egg yolk,[2] yielded on hydrolysis, in addition to oleic acid, acids with more than one double bond. From the egg lecithin, linolic and arachidonic acids were isolated; whereas from liver lecithins, arachidonic acid only was obtained. The fact that linolic acid has not as yet been isolated from the liver lecithins does not prove its absence, since neither the isolation of oleic nor that of arachidonic acid is quantitative.

The present note records the results of the analysis of brain lecithins as regards the character of their unsaturated fatty acids. It was found that the brain lecithins also contain besides oleic acid, acids with more than one double bond. Of these arachidonic acid was isolated in the form of its octobromo derivative.

For the present linolic acid could not be isolated. However, the question of its presence or absence in brain lecithins is not definitely answered.

EXPERIMENTAL.

Lecithin cadmium chloride was prepared free from amino-containing impurities by the method previously described.[3] All samples of amino-free cadmium salts of brain lecithin had an iodine value not greater than 45. The following analyses are typical.

No. 404. 0.2757 gm. substance absorbed 0.1238 gm. iodine, corresponding to an iodine number of 44.

[1] Levene, P. A., and Simms, H. S., *J. Biol. Chem.*, 1922, li, 285.

[2] Levene, P. A., and Rolf, I. P., *J. Biol. Chem.*, 1922, li, 507.

[3] Levene, P. A., and Rolf, I. P., *J. Biol. Chem.*, 1921, xlvi, 353.

No. 401. 0.2582 gm. substance absorbed 0.1071 gm. iodine, corresponding to an iodine number of 41.

These salts were hydrolyzed in the usual manner, and the iodine numbers of the whole mixed fatty acids determined.

0.2663 gm. substance absorbed 0.1261 gm. iodine, corresponding to an iodine number of 47.

The fractionation of these acids, dependent on the varying solubilities of the barium and lead salts, was carried out by the method described in the preceding paper.

The acid of lower unsaturation melted at 14°C. and had an iodine number of 87.

0.2616 gm. substance absorbed 0.2259 gm. iodine.
The iodine number of oleic acid is 90.

The more highly unsaturated fraction of these acids was brominated in the usual manner, and the precipitated bromide purified by extraction with ether. A grey powder weighing 1.5 gm. was obtained. When heated to 250°C., after very gradual darkening, it sintered without melting and analyzed as follows:

0.1020 gm. substance: 0.0302 gm. H_2O and 0.0932 gm. CO_2.
0.2020 "   "   : (Carius)   0.3190 gm. AgBr.
$C_{20}H_{32}O_2Br_8$.   Calculated.   C 25.43, H 3.42, Br 67.72.
                          Found.   " 24.91, " 3.31, " 67.22.

No linolic tetrabromide could be isolated from the mother liquor.

ON A POSSIBLE ASYMMETRY OF ALIPHATIC DIAZO COMPOUNDS. III.

By P. A. LEVENE and L. A. MIKESKA.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

In previous publications,[1,2] several observations were reported which seemed to point towards the existence of an optically active diazodiethyl succinate. The present note contains an additional proof in the same direction. Curtius[3] has shown that acting with diazoethyl acetate on benzoic acid, benzoylethyl glycolate is formed. It was expected that under similar conditions diazodiethyl succinate will form benzoyldiethyl malate. It was also expected that this substance could be prepared free from malic or fumaric esters which may form as by-products, since the former possesses a much higher boiling point. These expectations were realized. Acting with diazodiethyl succinate on benzoic acid, pure benzoyldiethyl malate was obtained with an optical activity

$$[\alpha]_D^{20} = + 0.22°$$

Under similar conditions diethyl malate acting on benzoic acid did not give even a trace of benzoyldiethyl malate.

Conversion of Diazodiethyl Succinate into Benzoyldiethyl Malate.— Crude diazodiethyl succinate (45 gm.) containing 9.23 per cent diazo nitrogen, was added in small portions to 30 gm. of melted benzoic acid. The temperature of the reaction mixture was maintained at 140°C., until the reaction was complete, which required about 8 minutes. It was then cooled, dissolved in ether, and washed three times with sodium carbonate solution and finally with distilled water. After drying the ethereal solution with anhydrous sodium sulfate, the ether was removed under dimin-

[1] Levene, P. A., and Mikeska, L. A., *J. Biol. Chem.*, 1920–21, xlv, 593.
[2] Levene, P. A., and Mikeska, L. A., *J. Biol. Chem.*, 1922, lii, 485.
[3] Curtius, T., *J. prakt. Chem.*, 1888, xxxviii, 427.

ished pressure and the residue extracted with petroleum ether to insure the removal of traces of benzoic acid. The residue was then fractionated under a pressure of about 135 mm. The first fraction boiled between 65 and 70°. The temperature then rose rapidly and the second fraction was collected between 130 and 149°C. The third fraction distilled between 150 and 160°C. The third fraction was redistilled under a pressure of 3 mm., and separated into two fractions, the first boiling at 146–147°C. and the second which was identified by analysis as benzoyl malate, boiled at 147–148°C.

0.1024 gm. substance: 0.0624 gm. H_2O and 0.2300 gm. CO_2.
$C_{15}H_{18}O_6$.   Calculated.   C 61.23, H 6.16.
                         Found.   " 61.25, " 6.81.

The substance had the following rotation:

$$[\alpha]_D^{20} = \frac{+\,0.22° \times 100}{1 \times 100} = +\,0.22°$$

The optical rotation of the original diazo compound was:

$$[\alpha]_D^{20} = \frac{+\,1.14° \times 100}{1 \times 100} = +\,1.14°$$

In another experiment 30 gm. of diazodiethyl succinate were treated with benzoic acid as in the preceding experiment. A fraction, boiling at 143°C. under 0.2 mm. pressure, was obtained. It had an optical rotation of

$$[\alpha]_D^{20} = \frac{+\,0.12° \times 100}{1 \times 100} = +\,0.12°$$

The percentage of carbon and hydrogen found, agreed well with the theoretical values.

0.1014 gm. substance: 0.0604 gm. H_2O and 0.0227 gm. CO_2.
$C_{15}H_{16}O_6$.   Calculated.   C 61.23, H 6.16.
                         Found.   " 61.27, " 6.61.

The original diazo compound had an optical rotation of

$$[\alpha]_D^{20} = \frac{+\,0.75° \times 100}{1 \times 100} = +\,0.75°$$

Action of Benzoic Acid on Diethyl Malate.—Malic ester (10 gm.) was gradually added to 10 gm. of melted benzoic acid exactly as in the preceding experiment. The temperature was maintained at 140–145°C. for 10 minutes. The excess of benzoic acid was removed by washing the ethereal extract with a solution of sodium carbonate. The extract was then dried, the ether removed, and the residue distilled. The entire amount distilled at 78–80°C. leaving only a trace of residue. In this case only about 1 gm. of substance was obtained whereas under similar conditions 45 gm. of diazodiethyl malate yield 42 gm. of combined distillates.

0.1012 gm. substance: 0.0716 gm. H_2O and 0.1824 gm. CO_2.

$C_8H_{14}O_5$.   Calculated.   C 50.52, H 7.36.
                  Found.   " 49.15, " 7.91.

THE METABOLISM OF INORGANIC SALTS.

I. THE ORGANIC ION BALANCE OF THE BLOOD IN PARA-THYROID TETANY.*

By ERWIN G. GROSS AND FRANK P. UNDERHILL.

(*From the Department of Pharmacology and Toxicology, Yale University, New Haven.*)

It is almost universally accepted that tetany and the metabolism of calcium are closely related. MacCallum and Voegtlin (1) in 1909 showed conclusively that in tetany in dogs, following the removal of the parathyroid glands there was not only an increased calcium excretion, but a marked decrease in blood calcium. The view that the parathyroid glands are intimately associated with the metabolism of calcium has gained many adherents, but the mechanism of this controlling function as yet is unknown.

Loeb (2), Ringer (3), and others have shown that the ratio of the inorganic ions of the blood is of far more importance than the absolute amount of each, in the maintenance of normal equilibrium of function, especially of the heart beat. If this is true, it becomes of interest to see whether the change of one of the elements of the blood will cause a compensatory shift in any of the other ions.

As far as can be found in the literature, the only study of this kind has been made by Greenwald, published in an article by Hastings and Murray (4). However, the results presented in that paper are incomplete. Such studies have only been made practicable in the last year or 2 by the perfection of methods which make it possible to determine the blood salts in a relatively small quantity of blood.

Recently Kramer, Tisdall, and Howland (5) have studied the inorganic salts in infantile tetany. Whether infantile tetany bears any relation to altered functions of the parathyroids does not receive very great support. Therefore, one is not justified in assuming that there are necessarily similar changes in salts in these two types of tetany, although both seem to be intimately associated with low blood calcium.

Perhaps the study of calcium in relation to tetany has been the most intensively studied, due to the observation of Sabbatani (6) in 1904, that calcium salts are related to the diminished irritability to electrical stimulation, while substances which reduce calcium caused increased irritability. The correlation of low calcium to increased irritability in tetany has given scientific foundation for the relational administration of calcium in the relief of spasmophilia. Quest (7) has found a reduced calcium content of the brain tissue of patients who had died from tetany. Neurath (8), Longo (9), and Cattaneo (10) have made studies in tetany and found a lowered calcium content, but the figures cited are so different from those of more recent investigators that one queries how much value can be placed upon them.

MacCallum and Voegtlin (1) and MacCallum and Vogel (11) in studies upon parathyroid tetany in dogs found not only an increased calcium excretion, but a marked fall in blood calcium. These authors further showed that the administration of calcium lactate temporarily relieved the tetany. Cooke (12) in 1910 was unable to find any marked increase in calcium excretion in tetany or a lowered calcium content of the brain in parathyroid tetany, and concludes that the parathyroids are not functionally related to calcium metabolism. Recently, however, Hastings and Murray (4) have obtained results very similar to those of MacCallum and Voegtlin (1) in the blood calcium.

Howland and Marriott (13) in 1917 showed that in infantile tetany the calcium of the serum decreased very materially while the phosphates and magnesium remained singularly constant. These observations have recently been reaffirmed in the same laboratory by Kramer, Tisdall, and Howland (5). The latter authors further found that sodium remains quite constant, but that potassium tends to increase as the calcium decreases. However, the change in potassium was not very marked, and they conclude that low calcium is responsible for infantile tetany.

Binger (14) in 1917 found that the injection of the alkaline phosphates caused tetany and also reduced the calcium, and advances the hypothesis that phosphates are responsible for the tetany. Greenwald (15), however, does not agree with this, and has shown that phosphates alone have no toxicity. He believes that the effect of the phosphates is due to a disturbance in the balance of the other elements.

To determine whether or not there is a disturbance in the balance of the elements in parathyroid tetany, it is necessary to make a complete study of the inorganic elements of the blood. With this view in mind we have attempted to follow the inorganic constituents through the various stages in parathyroid tetany, without special reference to the cause of tetany.

EXPERIMENTAL.

Dogs have been used as experimental animals. Normal values in the blood have been determined after a 48 hour fasting period except in one case, see Table V. The operations for removal of the entire thyroid—parathyroid structures were carried out under aseptic conditions and the animals fasted throughout the period of the experiment. Blood was obtained from the external jugular vein by aspiration. The chlorides were determined according to the Whitehorn (16) method, total phosphorus according to Bloor (17), and the calcium, potassium, sodium, and magnesium by the procedure of Kramer and Tisdall (18) for whole blood. Slight modifications were made in the method of potassium, as the potassium of dog blood is very much lower than in human blood, 1 cc. of the final acid filtrate was used instead of 0.2 cc. as called for in the original method. The proportions of the other reagents were not altered. Blank controls were determined upon all reagents and found to be sufficiently pure without necessitating any correction factors. Duplicate analyses were made in all cases. As the method of Kramer and Tisdall necessitates the weighing of the blood rather than the measuring, all the analyses are reported on a weight basis, and values are expressed per 100 gm. of whole blood rather than per 100 cc. as is the usual custom.

The Inorganic Ratios of the Normal Fasting Dog.

In a study of the ratio of the various ions it is necessary to establish the various ranges of the elements under normal fasting conditions and also after the operation to determine the effect of the anesthesia used in the operation. Analyses which tend to show the widest ranges of the various elements as determined have been selected and are presented in Table I. All analyses were made after 48 hours fasting, values expressed as mg. per 100 gm. of blood.

From the above analyses it will be seen that the inorganic elements under similar conditions are quite constant, the greatest variation occurring in the case of potassium. These values agree

TABLE 1.

The Inorganic Ions of the Blood during Fasting.

	Dog 1, ♀.	Dog 2, ♀.	Dog 3, ♂.	Dog 4.	Dog 5.	Dog 6.	Dog 7.
Cl$_2$..........	318.0	296.0	302.0	330.0	318.0	310.0	312.0
Total P.....	35.0	35.8	39.2	37.1	34.8	38.9	40.3
Ca..........	5.5	5.3	5.7	5.9	5.6	5.7	5.5
K..........	31.0	29.0	25.3	26.8	31.0	29.6	31.8
Na..........	312.0	305.0	301.0	310.0	315.0	313.3	312.0
Mg..........	5.8	5.2	4.9	4.9	5.9	5.6	4.6

fairly closely with those cited by Abderhalden (19), except in the case of magnesium, on which Abderhalden found much lower results while the correctness of the magnesium determinations were not checked against a known amount of magnesium, several analyses were made upon human blood with the same reagents and the values found agree very closely with those cited by Kramer and Tisdall.

Loeb (20) in 1915 found that to preserve normal irritability of nerves the ratio of $\dfrac{(Na) + (K)}{(Ca) + (Mg)}$ must be maintained fairly constant. In calculating the ratios of the monovalent ions to divalent ions of the seven analyses cited this ratio does approach a fairly constant figure, ranging between 29.2 to 33.3.

*Influence of Fasting and Ether Anesthesia upon the
Organic Ratios.*

In Table II the effect of starvation and of anesthesia will be
seen. This dog did not develop tetany after the removal of the
parathyroids, and remained alive, apparently normal for 2
months, at which time the animal was utilized for other experi-
mental purposes. The various elements before and 3 days after
the operation were practically unchanged within the limits of
experimental error. With the observed constancy of these
various ions, not only in the same individual, but also in
different individuals, any change of note in one of the elements
will readily effect the ratio.

TABLE II.

Influence of Fasting and Anesthesia upon the Inorganic Ions of the Blood.

Dog 8; male; weight 22.3 kilos; analyses after 48 hours fasting.

Date.	Chlorides.	Total phosphorus.	Calcium.	Potassium.	Sodium.	Magnesium.
1921						
Dec. 16.........	302	38.8	5.9	30.0	306	5.7
" 17.........			Anesthesia and operation.			
" 20.........	300	40.0	5.8	31.2	310	5.9

Considerable difficulty has been experienced in obtaining tetany
following the removal of the parathyroids. Over one-half of the
animals studied failed to develop any symptoms. In our exten-
sive experience with this type of investigation such a result is
unusual and difficult of explanation. Both thyroid and para-
thyroid glands were apparently removed in all instances, and it
must be concluded that these refractory animals had accessory
parathyroids, which were not imbedded in the thyroid tissue,
and thus failed to be removed in the operation.

*The Influence of Removal of the Thyroids and Parathyroids upon
the Inorganic Ratios of the Blood.*

Dog 9 was operated upon December 8th and on the 2nd day
developed symptoms of mild tetany (Table III).

The most striking changes in the elements here are the decrease
in calcium, and the increase in potassium and phosphorus. The

TABLE III.

The Influence of Removal of the Thyroids and Parathyroids upon the Inorganic Ion Balance of the Blood.

Dog 9; male; weight 10.8 kilos.

Date.	Per 100 gm. of blood.						Remarks.
	Chlorides.	Total phosphorus.	Calcium.	Potassium.	Sodium.	Magnesium.	
1921	mg.	mg.	mg.	mg.	mg.	mg.	
Dec. 6	306	36.8	5.9	22.4	307.6	5.6	Normal; taken 48 hrs. after last meal.
" 8							Complete removal of thyroid and parathyroid structures.
" 9	302	50.2	4.8	23.2	287.5	5.7	Dog showed tremors, increased temperature at 10.30 a.m. *Blood sample taken at 11.30 a.m.* Tremors stopped at 12.15 p.m. 10 30 p.m. dog apparently normal.
" 10	295.2	47.8	3.3	33.1	324.0	5 5	11 30 a.m. drank water very copiously. Increased respiration, tetany. *Blood sample taken at 12.00 m.* Shortly after bleeding, respiration returned to normal.
" 11	270.0	47.0	2.8	33 2	291.2	5 2	Severe tetany 9 a.m. *Blood sample taken 10 a.m.* followed by injection of 20 cc. of 5 per cent calcium lactate; 10.30 a.m. dog recovered. 9.30 p.m. severe tetany. Given 20 cc. of calcium lactate. 12 00 m., no relief, Cheyne-Stokes' breathing.
" 12	270 0	54.4	1.9	37.2	320 0	5.4	9.30 a.m. dog still in tetany; given 20 cc. of 5 per cent calcium lactate; 10 30 a.m. tetany practically disappeared; 11.30 a.m. slight tetany, labored breathing. *Blood sample at 6 p.m.* Condition about same. 10 p.m. dog dead, no convulsions, no frothing at mouth.

chlorides fell slightly, but the sodium and magnesium remained practically constant. The ratio of $\frac{(P)}{(Ca)}$ changes from 6.2 to 28.4 and the ratio of $\frac{(K)}{(Ca)}$ from 3.8 to 19. The ratio of the $\frac{(Na) + (K)}{(Ca) + (Mg)}$ changes from 29.0 to 48.5. These ratios are given for the normal and the final analysis attained preceding death, as this gives the greatest change in the constituents.

While the first injection of calcium lactate was not apparently effective, the second injection the following day quite promptly relieved the symptoms. The effect of calcium is of very temporary nature and the extra calcium injected soon leaves the blood stream as shown by the analysis made $8\frac{1}{2}$ hours after the exhibition.

Dog 10 developed tetany on the 3rd day after the operation (Table IV).

In this animal at the onset of tetany the blood calcium was reduced over 50 per cent while the other elements were little if any effected. The phosphorus was unchanged within experimental limits throughout the entire period. The ratio of $\frac{(K)}{(Ca)}$ changes from 4.5 to 22.2, and the ratio of $\frac{(Na) + (K)}{(Ca) + (Mg)}$ from 32.8 to 40.2. These ratios approach the ratios obtained in the preceding animal. It is impossible to draw any deductions from the ratios obtained just at the beginning of tetany, as usually the dogs had developed tetany during the night. However, comparing Dogs 9 and 10 when the first convulsive tetany appeared the ratios of $\frac{(K)}{(Ca)}$ are quite close ranging from 10 in Dog 5 to 10.2 in Dog 10. The ratio of the $\frac{\text{monovalent ions}}{\text{divalent ions}}$ is also remarkably close being 40.1 in Dog 9 and 40.2 in Dog 10.

That the injection of soluble calcium salts relieves the symptoms of tetany by increasing the blood calcium is apparent from the analysis. This analysis made 4 hours and 15 minutes after the injection of Ca lactate still showed a marked increase in calcium. The relief of tetany lasted over a considerably longer period than

TABLE IV.

The Influence of Removal of the Thyroids and Parathyroids upon the Inorganic Ion Balance of the Blood.

Dog 10; male; weight 13.7 kilos.

Date.	Per 100 gm. of whole blood.						Remarks.
	Chlorides.	Total phosphorus.	Calcium.	Potassium.	Sodium.	Magnesium.	
1922	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	
Jan. 3	290	40.5	5.2	23.5	313	5.04	Normal blood sample taken 2 days after last meal.
" 7							10.30 a.m. complete removal of thyroid tissue (4 parathyroids found).
" 8							Dog normal.
" 9							" "
" 10	275	39.6	2.8	28.8	281	4.9	8.30 a.m. severe convulsions, frothing at mouth, *blood sample taken.* Improved 9.45 a.m.
" 10	268	37.7	2.2	31.1	272	5.8	9.00 p.m. distinct tetanic twitching of legs.
" 11 4.45 p.m.	255	37.9	1.7	28.5	237	5.1	8.30 a.m. dog quiet. 1.00 p.m. beginning tetany. *4.45 p.m. marked tetany, blood sample drawn.* 5.45 p.m. severe convulsions. 6.28 p.m. given 20 cc. calcium lactate subcutaneously.
Jan. 11	263	38.5	3.3	29.5	240	5.7	9.00 p.m. dog resting quietly *(blood sample drawn).*
" 12							Dog resting quietly, 8.00 a.m. 2.00 p.m. mild tetany.
" 13	252	40.0	1.8	35.0	252	5.06	8.00 a.m. slight twitching. *2.00 p.m.* mild tetany, no increased respiration, *blood analyses.* 8.30 p.m. very increased tetany, given 20 cc. of calcium lactate.
" 14							Except for slight twitchings resting well. Blood count 7,256,000.

TABLE IV—*Concluded.*

Date.	Per 100 gm. of whole blood.						Remarks.
	Chlorides.	Total phos-phorus.	Calcium.	Potassium.	Sodium.	Magnesium.	
1922	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	
Jan. 15	250	41.8	1.3	27.3	229	5.1	*10.00* a.m. about same condition, *blood sample drawn.*
" 16–19							Jan. 16, 17, 18, 19 dog very weak with constant twitching, given food Jan. 19, ate all food.
" 20	264.5	40.2	1.4	31.0	231	5 1	Twitching markedly decreased; *blood sample taken.* Dog taken to basement, refused food; died Jan. 25 without any convulsions.

in the preceding case. While apparently the calcium is the only element effected the ratio of $\frac{(Na) + (K)}{(Ca) + (Mg)}$ at this analysis is 30, being approximately the normal ratio, while at the succeeding attack of tetany the ratio becomes 42, which is very close to the ratio of these ions at the first attack of tetany.

Dog 11 developed tetany the 3rd day following the operation (Table V).

This dog also showed a low calcium. The potassium in this dog did not rise beyond the range of experimental error. The normal potassium was very high, and perhaps this high value can be accounted for by the fact that the normal analysis was made directly after a meal. The dog never developed typical tetany, but showed a condition marked by stiffening of legs and violent breathing, finally dying of respiratory failure. The normal ratio of $\frac{(K)}{(Ca)}$ was 5.8 changing to 9.5 at first attack and the final ratio being 16.4. The ratio of $\frac{(Na) + (K)}{(Ca) + (Mg)}$ changed from 30 in the normal to 35.3 at first attack and finally reaching the

TABLE V.

The Influence of Removal of the Thyroids and Parathyroids upon the Inorganic Ion Balance of the Blood.

Dog 11; male; weight 19 kilos.

Date.	Per 100 gm. of whole blood.						Remarks.
	Chlorides	Total phosphorus.	Calcium.	Potassium.	Sodium.	Magnesium.	
1922	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	
Jan. 17	296	38 0	5.9	34.2	313	5 7	Normal blood sample drawn 30 min. after last meal.
" 21							Complete removal of thyroid and parathyroid structures.
" 22							Dog normal.
" 23							
10.30 a.m.	296	36 0	3 8	35.7	303	5 8	9 a.m. apparently well, 9.30 a.m. drank water copiously, 10 a.m. tetany in hind legs. *10.30 a.m. blood sample taken.* 11.00 a.m. dog improved, but slight twitching. 12 m. no twitching but dog restless. *10 p.m. no tetany, blood sample taken.*
10.00 p.m.	305	37.7	3 4	35 0	297	5 8	
Jan. 24	306	37.6	2.5	37.9	Lost.	5 2	9 a.m. same condition, *2.30 p.m.* after drinking copiously dog stiffened in cage and apparently had respiratory failure, artificially respirated and *blood sample taken.* Normal breathing returned after blood drawing.
" 25	297	42.0	2.2	36.3	313	5.5	Dog very restless, difficult breathing, slight twitching of legs. *Blood drawn at 11 a.m.* Dog died at 12.45 p.m. of respiratory failure, no convulsions.

value of 45.4. The ratio of the $\frac{\text{monovalent}}{\text{divalent}}$ ions at the first attack is considerably lower than in the other two dogs, but this may possibly be explained by the fact that the animal did not develop true convulsive tetany, as experienced in all the other animals.

Dog 12 developed tetany the 3rd day following the operation, showing a very active tetany in a short time (Table VI). In this case as in Dogs 9 and 10 the calcium dropped markedly and the potassium increased. The ratio of $\frac{(K)}{(Ca)}$ in the normal was 5.2 changing to 13.2 at the height and finally reaching the ratio of 17. The ratio of $\frac{(Na) + (K)}{(Ca) + (Mg)}$ ranged from 32.5 in normal to 43.9 at height and finally attaining the ratio of 51. The ratios of $\frac{(K)}{(Ca)}$ and $\frac{(Na) + (K)}{(Ca) + (Mg)}$ at height of tetany are higher than any of the preceding cases. This dog may possibly have had a previous attack during the night and recovered, which might also account for the sudden attack in the morning. No marked changes occurred in any of the other elements.

Dog 13, again developed tetany in 3 days and the ratio of $\frac{(K)}{(Ca)}$ varied from 5.12 in normal to 15.8 at height and the final ratio was 52.8. The ratio of $\frac{(Na) + (K)}{(Ca) + (Mg)}$ was 33.2 in normal to 49.8 at height, the final ratio being 52.8. All the ratios in this animal are again higher than those in the first three animals at the first severe tetany. This dog was in tetany in the morning when first observed, and may have had other attacks. This suggestion is strengthened by the fact that the drawing of blood did not benefit the condition, which is contrary to our observations with the most of the other animals. Bleeding at the beginning of tetany nearly always temporarily relieves the symptoms. Whether the ratios would have changed further is not known as the animal was bled to death after the last analysis.

As in the case of Dog 9, this animal showed a marked increase in the total phosphorus. Why such increases should be obtained

TABLE VI.

The Influence of Removal of the Thyroids and Parathyroids upon the Inorganic Ion Balance of the Blood.

Dog 12; female; weight 20 kilos.

Date.	Per 100 gm. of whole blood.						Remarks.
	Chlorides.	Total phosphorus.	Calcium.	Potassium.	Sodium.	Magnesium.	
1922	mg.	mg.	mg.	mg.	mg.	mg.	
Feb. 3	294	35.0	5.3	28.0	305	4.9	Normal taken 2 days after last meal.
" 6							Complete thyroid removal.
" 7							Dog normal.
" 8							" "
" 9 9.30 a.m.	267	35.0	2.8	37.0	310	5.1	9.00 a.m. dog apparently normal, drank water very copiously. 9.15 a.m. active tetany. *9.30 a.m. blood drawn.* 11.00 a.m. dog standing up, tetany about disappeared, breathing rapid. 11.45 a.m. severe tetany, chattering teeth, rapid respiration. 11.55 a.m. given 20 cc. 5 per cent calcium lactate subcutaneously. 1.45 p.m. still in convulsive tetany, rapid respiration, given 20 cc. 5 per cent calcium lactate. 3.00 p.m. tetany disappeared, respiration normal.
Feb. 9	292	35.0	2.7	32.3	309	5.2	*5.00 p.m. blood sample taken.* 6.00 p.m. dog apparently normal.
" 10	270	34.8	2.8	32.0	274	4.5	*9.00 a.m.* dog in severe tetany, *blood sample taken.*
" 11	290	34.5	2.0	34.0	298	4.6	8.30 a.m. dog in tetany of more or less severe spasmodic attacks. *4.30 p.m. blood sample taken;* spasmodic tetany.
" 12							No blood sample taken. Dog about the same.
" 13							Dead. No convulsions.

in some cases and not in others is not understood.   In the results of Greenwald published by Hastings and Murray (4) he has found increases in phosphorus in all the cases cited.   Our results do not entirely agree with those of Greenwald.

While it is well known that calcium exists entirely in the serum, and potassium chiefly in the cells, it is not easy to explain these changes on the ground that the blood has become more concen-

TABLE VII.

The Influence of Removal of the Thyroids and Parathyroids upon the Inorganic Ion Balance of the Blood.

Dog 13; female; weight 12 kilos.

Date.	Per 100 gm. of whole blood.						Remarks.
	Chlorides.	Total phosphorus.	Calcium.	Potassium.	Sodium.	Magnesium.	
1922	mg.	mg.	mg.	mg.	mg.	mg.	
Mar. 7	310	36.8	5.8	26.1	330	4.9	Normal after 48 hrs. fasting. Thyroid and parathyroids removed.
" 8							*Dog normal.
" 9							" "
" 10 8.30 a.m.	278	50 0	2.4	38.0	320	4.8	7.30 a.m. slight twitching movements. 8.00 a.m. rapid breathing and tetany. 8.30 a.m. blood sample drawn.
Mar. 10 5 p.m.	289	55.7	1 9	38.6	321	4.9	No especial benefit from bleeding, continued tetany. Blood sample taken at 5 p.m. and dog bled to death.

trated.   While neither blood solids nor hemoglobin have been followed in these experiments, it has been shown in this laboratory by Underhill and Nellans that the hemoglobin values remain fairly constant in parathyroid tetany.   Further proof that this cannot be the explanation is the fact that the chlorides, sodium, and magnesium remain practically constant, and also phosphorus in the majority of cases.

Metabolism of Inorganic Salts.   I

SUMMARY OF THE RATIOS OBTAINED IN TETANY.

That the symptoms of the increased irritability and tetany are closely allied to the disturbed ratios of the various ions will be seen in Table VIII.

From this table the results suggest that changes in the ratios are more important than the absolute changes in any one of the elements. When the ratio of the $\frac{monovalent}{divalent}$ ions reaches 40 and above the animals always show convulsive tetany, while the ratio $\frac{(K)}{(Ca)}$ of 10 or above is accompanied by convulsive tetany.

TABLE VIII.

Ion Ratios in Tetany after Removal of Thyroids and Parathyroids.

Period.	Ions.	Dog 9.	Dog 10.	Dog 11.	Dog 12.	Dog 13.
Normal..............	$\frac{(K)}{(Ca)}$	3.8	4.5	5.8	5.2	5.1
Height...............		10 0	10.2	9.5	13.2	15.8
Death...............		19.0	22.2	16.4	17.0	23.1
After Ca injections succeeding tetany.......		9.0				
		19.5				
Normal..............	$\frac{(Na)+(K)}{(Ca)+(Mg)}$	28.7	32.8	30.0	32.8	31.4
Height...............		40 1	40.2	35.3	43 9	49.8
Death...............		48.5	40.2	45.4	51.0	52.8
After Ca injection succeeding tetany.......		30.0				
		42.0				

DISCUSSION.

From the preceding results it is apparent that there is a marked change in certain of the blood constants, especially in the case of calcium and potassium following parathyroid tetany. Whether the removal of the parathyroids is directly responsible for these changes, or whether the change in the blood salts is a secondary manifestation cannot be inferred from the data. However, we are of the opinion that the parathyroids function in some manner or other in the detoxication of some toxic products which probably affects the calcium. Recently Luckhardt and Rosenbloom

(21) have shown that by continuous intravenous injections of calcium-free Ringer's solution, thereby keeping up a brisk diuresis, they were able to maintain tetany dogs for 51 days. This would immediately point to some toxic substances being washed out of the blood. Paton and Findlay (22) have ascribed this toxic product to guanidine, and were able to produce typical tetany in dogs by injection of this substance.

It has long been observed, as our own experiments show, that at least in the early part of tetany, the mere expediency of drawing blood will temporarily alleviate the symptoms of tetany. It is very difficult to explain such a phenomenon, unless we assume a toxic product in the blood stream.

In the relation of calcium to potassium a rather remarkable change may be observed. It is a well recognized fact that potassium is antagonistic to calcium. There are no readily available stores of calcium to be called upon, while the various cells of the body store large amounts of potassium, which could be easily called into use. The more potassium poured into the blood, the greater becomes the disturbance in the equilibrium of the ions. If the relation of the ratios between these two elements at the time of the first attack of severe tetany are of any significance in relation to the conception of these symptoms, then a further increase in the ratio of potassium to calcium must be more detrimental to the organism. That the change in potassium as well as calcium perhaps has an apparent importance is highly probable. Kramer, Tisdall, and Howland (5) have found increases in potassium in infantile tetany. MacCallum and Voegtlin (1) in studying the effect of various salts upon tetany found that the injection of potassium acetate did not relieve the symptoms, but if anything tended to increase the tetany, while it required a greater subsequent injection of calcium lactate to relieve these animals. While these experiments of the latter authors are of doubtful significance they at least furnish an interesting speculation. That this peculiar relation of calcium and potassium is not an idiosyncrasy of tetany alone has been shown in this laboratory. By the injection of sodium oxalate the calcium of the blood can be greatly decreased, while the potassium rises, even to a greater amount than that obtained in tetany. With these facts before

us, it is quite possible that calcium and potassium are very intimately related in their metabolism, and when the equilibrium existing between them is disturbed grave consequences follow.

CONCLUSIONS.

1. The blood salts in dogs under normal fasting conditions are practically constant.

2. Parathyroidectomy chiefly disturbs the ratio between calcium and potassium.

3. The inception of tetany obtains with a definite change in the ratio of calcium to potassium.

4. The hypothesis is suggested that both low calcium and high potassium are factors in the production of the increased irritability.

BIBLIOGRAPHY.

1. MacCallum, W. G., and Voegtlin, C., *J. Exp. Med.*, 1909, xi, 118.
2. Loeb, J., *Am. J. Physiol.*, 1899-1900, iii, 327.
3. Ringer, S., *J. Physiol.*, 1880-82, iii, 380; 1883-84, iv, 29. Ringer, S. and Buxton, D. W., 1887, viii, 15.
4. Hastings, A. B., and Murray, H. A., Jr., *J. Biol. Chem.*, 1921, xlvi, 233.
5. Kramer, B., Tisdall, F. F., and Howland, J., *Am. J. Dis. Child.*, 1921, xxii, 560.
6. Sabbatani, L., *Mem. Accad. Reale Sc. Torino*, 1904, liv, series 2, 459.
7. Quest, R., *Jahrb. Kinderh.*, 1905, lxi, 114.
8. Neurath, R., *Z. Kinderh.*, 1910, i, 3.
9. Longo, A., *Policlinico*, 1910, xvii, sez. med., 495.
10. Cattaneo, C., *Pediat.*, 1909, vii, series 2, 414.
11. MacCallum, W. G., and Vogel, K. M., *J. Exp. Med.*, 1913, xviii, 618.
12. Cooke, J. V., *J. Exp. Med.*, 1910, xii, 45.
13. Howland, J., and Marriott, W. McK., *Quart. J. Med.*, 1917-18, xi, 289.
14. Binger, C., *J. Pharmacol. and Exp. Therap.*, 1917-18, x, 105.
15. Greenwald, I., *J. Pharmacol. and Exp. Therap.*, 1918, xi, 281.
16. Whitehorn, J. C., *J. Biol. Chem.*, 1920-21, xlv, 449.
17. Bloor, W. R., *J. Biol. Chem.*, 1918, xxxvi, 33.
18. Kramer, B., and Tisdall, F. F., *J. Biol. Chem.*, 1921, xlviii, 223.
19. Abderhalden, E., *Z. physiol. Chem.*, 1898, xxv, 88.
20. Loeb, J., *J. Biol. Chem.*, 1915, xxiii, 423.
21. Luckhardt, A. B., and Rosenbloom, P. J., *Proc. Soc. Exp. Biol. and Med.*, 1921-22, xix, 129.
22. Paton, D. N., and Findlay, L., *Quart. J. Exp. Physiol.*, 1916, x, 203.

STUDIES OF GAS AND ELECTROLYTE EQUILIBRIA IN BLOOD.

I. TECHNIQUE FOR COLLECTION AND ANALYSIS OF BLOOD, AND FOR ITS SATURATION WITH GAS MIXTURES OF KNOWN COMPOSITION.

By J. H. AUSTIN, G. E. CULLEN, A. B. HASTINGS, F. C. McLEAN,
J. P. PETERS, and D. D. VAN SLYKE.

(From the Hospital of The Rockefeller Institute for Medical Research.)

INTRODUCTION.

Recent articles by L. J. Henderson (1920, 1921), Parsons (1919), Van Slyke (1921, b; 1922), and A. V. Hill (1922) have summarized the present state of our knowledge concerning the interaction of oxygen, carbon dioxide, hemoglobin, chlorides, and other constituents involved in the respiratory function of the blood and in the maintenance of its neutrality and osmotic pressure. The field in its present state has been developed from the blood gas studies of Bohr, Haldane, Barcroft, and their collaborators, and the investigations of the blood electrolytes dating from Zuntz through Hamburger and Gürber to present investigators (*e.g.* L. J. Henderson, 1908; Van Slyke and Cullen, 1917; Adolph and Ferry, 1921; Fridericia, 1920; Doisy, Eaton, and Chouke, 1922; Barcroft, Bock, Hill, Parsons, Parsons, and Shoji, 1922).

The reactions known to be involved in the respiratory changes of the blood, and the accompanying shifts of gases and acids between plasma and cells, are in part indicated qualitatively by the accompanying diagram (Fig. 1).

All six reactions are forced from left to right by increase in H_2CO_3, which results in formation in the plasma of bicarbonate from two sources (Reactions 1 and 2), and in the cells from two other sources (Reactions 5 and 6).

Plasma.

Red cell wall.

Cell.

(1) $H_2CO_3 + Na\ Protein \leftrightarrows H\ Protein + NaHCO_3$

(2) $H_2CO_3 + NaCl \leftrightarrows NaHCO_3 + HCl$ ⟵ $\longrightarrow HCl$ ⟵ (3) $HCl + K_2HPO_4 \leftrightarrows KH_2PO_4 + KCl$

(4) $2\ HCl + 2KHbO_2 \leftrightarrows 2\ KCl + \begin{cases} HHbO_2 \\ HHb + O_2 \end{cases}$

H_2CO_3 ⟵ $\longrightarrow H_2CO_3$ ⟵ (5) $H_2CO_3 + K_2HPO_4 \rightarrow KHCO_3 + KH_2PO_4$

(6) $2\ H_2CO_3 + 2\ KHbO_2 \leftrightarrows 2\ KHCO_3 + \begin{cases} HHbO_2 \\ HHb + O_2 \end{cases}$

O_2 ⟵ $\longrightarrow O_2$ ⟵

Fig. 1.

The displacement of base from combination with hemoglobin by HCl (Reaction 4) or H_2CO_3 (Reaction 6) results in a decrease in the oxygen bound by hemoglobin (last steps of Reactions 4 and 6), because when alkali hemoglobinate loses part of its alkali it also loses part of its affinity for oxygen.

On the other hand, increase of O_2 (from the lungs) forces Reactions 4 and 6 backwards, and thereby through Reactions 6 and 2 sets H_2CO_3 free.

By the reactions outlined influx of oxygen sets H_2CO_3 free and thereby helps the blood throw off in the lungs its overload of CO_2; while in the capillaries influx of CO_2 sets oxygen free from combination and thereby makes it more readily available to the tissues.

It is apparent from the diagram that every reaction affects every other reaction. In a given blood the O_2 and the H_2CO_3 concentrations are the two variables that are directly changed by respiration, and their alterations govern the accompanying changes in all the other variables, which are dependent on them. As L. J. Henderson has pointed out (1921), when we have in a given blood only two independent variables to deal with, by determining the relationship of each other variable to these two, or to any given two within the system, the quantitative interrelationships among them all may be determined. This may be accomplished either algebraically, or, more simply, by a two dimensional diagram such as Henderson has used (1921), or by an alignment chart. (The pH, although not indicated on our diagram, is also one of these dependent variables, since its value is set by the $BHCO_3$ and H_2CO_3 according to Hasselbalch's (1917) equation $pH = pK' + \log \frac{[BHCO_3]}{[H_2CO_3]}$).

It appears that the chief substances and reactions involved in the respiratory changes of the blood are probably known; and that Henderson has solved the mathematical problem of expressing by a practicable method the many relationships involved.

One cannot, however, read the recent theoretical papers quoted at the beginning of this paper without being struck by the present inadequacy of experimental data sufficiently accurate and complete to permit within definable limits of error the formulation of the

quantitative relationships involved.[1] Such data require simultaneous observations of several changes with technique of a precision gauged in detail to meet the requirements of the problem.

Preliminary experiments leading towards the systematic determination of data on the various factors in the system were begun by McLean, Murray, and L. J. Henderson (1920) in Henderson's laboratory. The magnitude of the task made a coordinated division of it between at least two laboratories advisable, and the present series of papers from this laboratory is the partial result of such a division.

The problem of the determination of CO_2 and O_2 tensions by analyses of the gas phase was already solved by Haldane's apparatus for air analysis. We used this apparatus with the single 4-way stop-cock introduced by Yandell Henderson (1918).

The methods available for the determination of blood gases were less satisfactory. Fig. 2 is constructed from observations collected from the literature by Peters, Barr, and Rule (1920), and presents in graphic form the relations which appear to obtain between the carbon dioxide tension, the carbon dioxide concentration, and the hydrion concentration in average normal human blood. On this figure have been introduced carbon dioxide absorption curves (a), of completely oxygenated blood; (b), of completely reduced blood (located at the distance above the curve of oxygenated blood indicated by the results of Christiansen, Douglas, and Haldane (1914)); and (c), of plasma from the oxygenated blood. A comparison of these curves shows the magnitude of the changes which it is necessary to analyze.

The large unblocked rectangle (A) represents an estimation of the maximum combined error involved in the determination of the carbon dioxide absorption curve by a technique employed recently by Peters, Barr, and Rule (1920). The error is indicated in terms of pH, of volumes per cent of carbon dioxide in the blood, and of millimeters of carbon dioxide tension. We believe that the procedure employed in these experiments was as accurate as that used by previous workers. Nevertheless, the rectangle representing the possible cumulative error covers 40 per cent of the difference

[1] The quantitative relationships between Reactions 1 and 2 appear satisfactorily established in a paper by Doisy, Eaton, and Chouke (1922), which appears as this paper goes to press.

Fig. 2.

A = Maximum combined error estimated to exist in method used by Peters and Barr for the determination of the CO_2 absorption curve.

(2.5 vol. per cent)

B = Error ascribed by Hasselbalch to the electrometric method of determining pH. (0.02)

C = Maximum error of earlier methods of measuring the carbon dioxide content of blood. (1.0 vol. per cent)

D = Error of present electrometric pH measurements. (0.01)

E = Small black square in center represents variations in duplicate determinations of CO_2 content by improved method now employed.

(±0.1 vol. per cent)

"This graph was intended only to show errors in measurements and not absolute values. If used to read pH from observed CO_2 content of blood or plasma and CO_2 tension and if one accepts pK' for whole blood = 6.15 and pK' for serum = 6.10, then a correction for whole blood of +0.04 pH must be added and for plasma or serum of −0.04 pH. The diagram would otherwise appear to indicate that the pH of whole blood is 0.08 lower than its true plasma at the same CO_2 tension. The pH of whole blood, however, _____ its true plasma at the same CO_2 tension."

between the carbon dioxide content of completely reduced and that of completely oxygenated blood. The cross-hatched rectangle (C) represents the limits of variation in duplicate determinations of the blood carbon dioxide by the method presented by Van Slyke in 1917 which was used by Peters, Barr, and Rule. These limits are of the same magnitude as those usually ascribed to the Barcroft and Haldane methods. The difference between the clear rectangle (A) and the cross-hatched rectangle (C) indicates the sum of the errors introduced by the manipulations which preceded the analyses. Although these errors are only estimated, we believe the estimates are approximately correct.

By improvements in apparatus and procedure for blood gas determinations (Van Slyke and Stadie, 1921), it is now possible to obtain consistently duplicate carbon dioxide readings with an average variation from the mean of $\pm$ 0.1 volume per cent or $\pm$ 0.05 millimol, a value represented by the small solid black square. A more recent improvement[2] has reduced the *maximum* variation in results by a skilled analyst, to $\pm$ 0.05 millimol of either CO_2 or oxygen. This variation is of about the same magnitude as that entailed in the volumetric measurement of blood in a pipette calibrated to deliver 1 cc. of water.

Variations in chloride estimations may be reduced to $\pm$ 0.1 millimol by the application of the method of Austin and Van Slyke (1920, 1921) to sufficiently large samples of blood

The analytical errors having thus been reduced, it remained to develop a procedure for preliminary treatment which could be reproduced so accurately that two specimens of blood subjected to it would not differ in composition from each other, or from their common curves, by more than the analytical errors. It is the purpose of this paper to present a technique for the preliminary treatment of blood which will meet these requirements.

Table I presents the steps in the procedure of an ordinary experiment, and the factors which are active in the production of error.

[2] The principle of this apparatus was published last year in a preliminary note (Van Slyke, 1921, *a*). The details will appear shortly by Van Slyke and Neill in this *Journal*.

TABLE I.

Factors Involved in Determining Gas and Electrolyte Equilibria in Blood.

Steps in procedure.	Sources of error.
The drawing, preparation, and preservation of blood............ 1 The saturation of blood at a desired temperature with a desired gas mixture............ 2	Hemolysis. Formation of non-volatile (lactic?) acid.
Determination of the exact composition of the gas mixture at equilibrium.................... 3	Change of equilibrium conditions during separation of gas and liquid phases.
The delivery of blood from the tonometer into a receiving vessel ... 4 Separation of serum or plasma ... 5	Change of gas content of blood by exposure to air or oil.
Preservation of blood, plasma, or serum for analysis.............. 6	Change of gas content by exposure. Formation of non-volatile acid (whole blood only). Formation of CO_2 and consumption of O_2 (whole blood only).
Analyses of blood, plasma, or serum........................ 7	Change of gas content by exposure during transfer of sample from container to apparatus for analysis. Uneven mixture of cells in whole blood at moment of measuring sample. Limit of accuracy of methods for analysis of gas and liquid phases.

Sources of Error.

1. Hemolysis.—Hemolysis changes the distribution of gases and electrolytes between cells and plasma. We avoided hemolysis by careful handling, and by using for most of our animal experiments horse blood, the cells of which are less fragile than those of dog blood. In the case of dog blood, we generally used serum in place of plasma, when determinations on the cell-free fluid were required.

2. Formation of Non-Volatile Acid in Blood.—Christiansen, Douglas, and Haldane (1914) showed that if defibrinated human whole blood is kept at 37° its carbon dioxide capacity falls by as much as 2 volumes per cent in a half hour, apparently because of the formation of non-volatile acid.   Peters, Barr, and Rule (1920) observed a similar acid formation in human blood, both defibrinated and oxygenated, but found that when the blood was allowed to stand at room temperature after being drawn it showed no significant changes during the 1st hour.

The formation of acid is confined to the cells, since it does not occur in separated plasma.   The time of onset varies in bloods of different species.   Dog blood has been observed to change measurably in an hour at room temperature, while horse blood has shown no measurable change in several hours.   In a paper which appears as this goes to press Evans (1922) suggests that the non-volatile acid is formed by glucolysis, since its rate of formation parallels that of glucose disappearance.   He finds that the acid formation is accelerated by loss of CO_2 (increase in pH) and is retarded by the addition of 0.1 per cent of sodium fluoride.

Up to the present we have avoided error from acid formation by working with horse blood, and by chilling the blood to zero whenever any time was allowed to elapse between the withdrawal of the blood and its exposure to a gas mixture or between this exposure or centrifugalization and analysis.   We have also used uniform periods for saturation at 38°, so that if acid formation should occur it would be relatively constant in different blood samples.   As a control of this factor, in experiments which involved a long series of exposures of samples of a given blood, we have repeated the conditions of the first exposure on the last of the series. Even when the chilled horse blood was permitted to stand some hours between the first and last exposures, no change was noted.

3. Formation of CO_2 and Consumption of O_2 by Metabolism of Whole Blood.—It has been shown by Harrop (1919) that, by this process, oxalated normal human blood loses 0.1 to 0.4 volumes per cent of oxygen in 6 hours at 38°.   In horse blood we have found no significant changes at room temperature in an hour, which was the maximum time that blood was allowed to stand unchilled between withdrawal or saturation and analysis.

4. Uniform Mixtures of Cells and Plasma.—The necessity of obtaining a uniform mixture before samples are taken for either analysis or saturation is especially great in horse blood, because the cells settle with unusual rapidity.   The uniformity of mixture

FIG. 3.

attained by our procedures was controlled by oxygen capacity determinations, and the procedures were regulated accordingly. The tubes in which blood was kept over mercury (Tube *J*, Fig. 3), for example, were inverted twenty times immediately before a sample was withdrawn.

Change of Equilibrium Condition During Separation of Gas and Liquid Phases.—Changes in temperature or pressure, such as may result when the tonometer is removed from the bath, may so disturb conditions of gaseous equilibrium existing in the tonometer as to render it impossible to obtain results sufficiently accurate for the purposes of the problem outlined above.

In the procedure we have usually used ("First saturation method" outlined below), the gas and liquid phases, in a state convenient for analysis, have been separated while the tonometer was still in the water bath by a mechanical device which avoided changes of temperature and pressure. In a second process, used for large amounts (over 30 cc.) of blood, the initial composition of the gas phase was fixed with especial care, and the amounts of CO_2 and O_2 taken from or given to it by the blood during saturation were calculated from accurate analyses of the blood before and after the saturation. Consequently analysis of the gas phases was avoided.

Collection and Preparation of Blood. (a). *For Saturation without Previous Analysis.*—When blood is to be exposed to known gas mixtures prior to all analyses, rigid precautions to avoid changes in gas content before exposure are, of course, not required. We have, however, avoided gross losses of carbon dioxide, such as might possibly cause irreversible changes, by drawing the blood under oil in cylinders arranged as described by Van Slyke and Cullen (1917, Fig. 1).

When oxalated blood was desired, potassium oxalate of tested neutrality was placed in the receiver in the proportion of 0.3 gm. to 100 cc. of blood. A saturated solution of neutral potassium oxalate was spread in a film on the walls of the receiver and dried by an air current. The neutrality of the oxalate was tested by the addition of phenol red to a diluted sample. Some samples of oxalate are alkaline. To such, oxalic acid was added until the pH was 7.4.

When defibrinated blood was required the oxalate was omitted, and the blood was defibrinated by stirring under oil with a rod. The blood was then filtered under oil through gauze.

(b). *Collection and Centrifugation of Blood for Analysis.*—When it was necessary to ascertain the gas content of the blood as drawn, as well as after exposure to a known gas mixture we either drew it

under oil by the above procedure, or used the apparatus shown in Fig. 3. When small samples (10 to 20 cc.) were drawn under oil, the precaution was taken to use rubber and glass tubes (the latter Pyrex) of only 2 mm. diameter for the blood to pass through, so that it would form a solid column without bubbles.

The receiving vessel, J, coated inside with oxalate, is connected with the mercury bulb M and the 3-way cock H bearing the needle. J and its connections up to H are filled with mercury, a few drops of which are wasted through F. The needle is then inserted into the blood vessel, and a few drops of blood are permitted to escape from F, in order to abolish the air-space remaining in the connecting tube. By turning H (with M in the lower position shown in Fig. 3) the needle and collecting vessel are directly connected and the blood is drawn into the latter. We have used tubes of the type J of from 10 to 200 cc. capacity.

As soon as the blood has been drawn J is inverted a number of times in order to insure mixing of the blood and oxalate, a process which is assisted by the mercury that remains. The leveling bulb M is kept at the upper level, in order to keep positive pressure on the blood sample.

Sampling Blood.—The cells and plasma are thoroughly mixed immediately before sampling by repeatedly inverting J. The leveling bulb being in the upper ring, the pipette P is inserted into the rubber tube outlet of J, the pinch-cock is opened, K is opened, and blood is allowed to flow up into the pipette.

Centrifugation without Loss of CO_2.—We have used centrifuge tubes of the type L in Fig. 3, similar to that described by Parsons (1919). A Pyrex centrifuge tube is fitted with a 1-hole stopper with a flange. Mineral oil is placed in the open tube, and the blood is allowed to flow under the oil and displace all but the last drops of oil from the tube. The stopper is then inserted, forcing out the remaining oil through the hole in the rubber. The glass plug is inserted in the hole, and the tube is ready for centrifugation.

After centrifugation the glass plug is removed, and a pipette containing oil is inserted in the hole. When the stopper is removed the oil flows over the surface of the plasma and prevents the escape of CO_2 during transfer of the plasma to the sampling tube J. For the protection needed, mineral oil is adequate, since it is in contact with the blood only a short time during which the blood is not agitated.

As an alternative to stoppering the centrifuge tube as above described, the surface of the blood may be covered, after most of

the mineral oil has been removed, by a layer of melted paraffin (melting point 40–45°) which need not exceed 5 mm. in thickness. After centrifugation, a little mineral oil is poured over the paraffin. A transfer tube or pipette may then be inserted through or along side the paraffin to remove the plasma. This technique is useful if the amount of blood is too small to fill the centrifuge tube completely.

The effects of variations in treatment on the loss of CO_2 from solutions containing $NaHCO_3$ and H_2CO_3 in about the concentrations found in normal human plasma are illustrated in Table II.

9. Separation of Plasma from Centrifugated Blood.—The capillary tube I (Fig. 3) is connected to J, clamped in position as shown, and J and I are completely filled with mercury. The stopper is then removed from the centrifuge tube, and the latter is held so that the tip of I extends below the protecting layer of oil. With M at the lower level, K is opened and the plasma withdrawn into J. The pinch-cock is then placed in position on the outlet tube of J, I is removed, and M placed in its upper support. Samples for analysis are withdrawn as described above.

10. Preparation of Mercury and Apparatus Used for Collecting and Centrifugating Blood.—Commercial "redistilled" mercury before it was used was washed by falling from a capillary through a 1 meter column of 10 per cent nitric acid, and then through columns of distilled water. After contact with blood or serum, mercury was washed twice by shaking with distilled water, and was then passed through the nitric acid and water towers. Before use it was tested for the presence of alkali or acid by shaking a few cc. in a test-tube with water containing brom-cresol purple and phenol red. Glassware and rubber for use in contact with blood were cleaned, thoroughly rinsed with distilled water, and dried by drainage and evaporation at room temperature. When rapid drying of tonometers was necessary, they were rinsed with alcohol and ether of tested neutrality and dried in an air current.

Saturation.

We have used two processes to bring blood into equilibrium with gas mixtures of known composition. In both of them measured volumes of the gases were introduced with the blood into tonom-

eters, which were then revolved in a water bath at 38° until equilibrium was attained. In one process, however, the calculation of final gas tensions was based on direct gas analyses, the gas and

TABLE II.

Loss of CO_2 from $BHCO_3$, H_2CO_3 Solutions during Standing and Centrifugating.

Change in pH was used as an index of CO_2 loss. A 0.03 M $NaHCO_3$ solution containing phenol red was saturated at 38° with air containing CO_2 at 50 mm. tension. 20 cc. portions of the solution were then transferred under oil to centrifuge tubes and treated as outlined.

No.		Initial.	Final.	Change.
		pH	pH	pH
1	An unstoppered 2 cm. (diameter) tube containing 10 cc. solution under a 1 cm. layer mineral oil was allowed to stand for 1½ hrs..........	7.35	7.35	0.0
2	An unstoppered 2 cm. tube containing 10 cc. solution under a 4.5 cm. layer mineral oil was allowed to stand for 1½ hrs...............	7.35	7.37	+0.02
3	An unstoppered 2.5 cm. tube containing 25 cc. solution under a 1.5 cm. layer mineral oil was centrifugated for 1 hr. and then allowed to stand for ½ hr...............	7.36	7.44	+0.08
4	A stoppered 2.5 cm. tube completely filled with 25 cc. solution, under 27 cc. mineral oil, was centrifugated for 1 hr. and then allowed to stand for ½ hr...............	7.36	7.52	+0.16
5	An open 2.5 cm. tube containing 25 cc. solution under a 1 cm. layer of solid paraffin was centrifugated for 1 hr. and then allowed to stand for ½ hr...............	7.35	7.37	+0.02
6	A stoppered 2.5 cm. tube completely filled with 50 cc. solution and containing no oil was centrifugated for 1 hr. and then allowed to stand for ½ hr...............	7.36	7.36	0.0

blood being separated at the end of saturation and analyzed separately. In the other process, which was usually employed when volumes of blood exceeding 30 cc. were used, direct analyses

of the gas phase were not made. The gas mixture was made up with especial accuracy, and the changes in its O_2 and CO_2 content during saturation of the blood were estimated from the amounts of these gases given off or taken up by the blood, which was analyzed both before and after its exposure to the gas mixture. With this procedure, separation of the gas phase in condition for analysis after saturation was unnecessary.

The oxygen (commercial) used was tested for purity by absorption with pyrogallol. The CO_2 from a Kipp apparatus, was tested by absorption with KOH solution. The hydrogen, sometimes commercial, sometimes from a Kipp apparatus, was analyzed for oxygen by absorption with pyrogallol in a Haldane apparatus. Nitrogen (commercial) was analyzed for contaminating oxygen in the same manner. When air was introduced it was freed of CO_2 by passage through a tower filled with moist sodium hydroxide "shells". This treatment reduced the CO_2 content of laboratory air to 0.01 per cent or less. In detail the two procedures for saturation of the blood were carried out as follows.

First Saturation Method. Final Tensions Determined by Analysis of Gas Phase.—The tonometer used (No. 1, Fig. 3) was a modification of a form introduced by Fridericia (1920), and consisted of a relatively small vessel (5 to 30 cc.); just sufficient to hold the blood, connected by a rubber tube of 6 mm. inner diameter to a larger vessel (of about 300 cc.). A length of about 3 cm. of rubber tubing was left between the glass parts. In order to fill the tonometer with the desired gas mixture, the tonometer was connected with the gas manifold at T (Fig. 3), and the air was drawn out through E. When the saturation was to be performed at an oxygen tension lower than that of air, nitrogen or hydrogen was then twice admitted, withdrawn as completely as possible, and readmitted. It was finally again drawn out, this time only until the pressure was reduced to about half an atmosphere. From the gas burettes through A and C sufficient CO_2 and O_2 were then admitted to give the desired tensions of these gases (see Equations 3 and 5 below). Finally, nitrogen or hydrogen was admitted until atmospheric pressure was attained.

The tonometer was then rotated horizontally in the bath, the blood being so distributed between the two chambers that the ratio of blood to gas volume was about the same in both. (A

rotating rack in the water bath held four tonometers at once.) The cock of the tonometer was opened near the surface of the water several times at intervals of a few minutes, until no more gas bubbles escaped. At each opening the tonometer was held so nearly upright that all the blood drained into the lower chamber; it was redistributed before the tonometer was clamped back into place.

The time required to attain equilibrium between a gas mixture and blood is dependent on the time necessary for the tonometer contents to reach bath temperature, on the relation of gas volume to liquid surface, on the invasion coefficient of the gas (Bohr, 1905, b), and, also, when the gases combine with substances in the blood, on the rate of combination. Under the conditions above outlined, about 15 minutes sufficed for the production of equilibrium when only CO_2 changes were involved, while 30 to 40 minutes were required when the oxygen tension of the blood was greatly reduced by the process.

Because the Δ of Equation 3 can be estimated only approximately, the gas tensions obtained at the end of a single saturation can be only approximately predetermined. Since the exact final tensions are accurately found by gas analyses, however, approximate predetermination of them is usually all that is needed (when, for example, data are being obtained for a curve).

When, however, it was desired to bring the blood gases to an exactly predetermined final tension, exposure in the tonometer was repeated once; and, when large tension changes were involved, twice. After the first exposure the tonometer was held upright in the bath until all the blood was in the lower chamber, which was then clamped off from the upper. The gas mixture in the latter was then drawn out and replaced as described above, with a mixture of the exact tension desired.

When the final saturation was complete, the tonometer was held upright in the bath until the blood had drained as completely as possible into the lower chamber. The latter was next separated from the upper chamber by screwing two clamps upon the rubber connecting tube. One clamp was attached near each glass part, that nearest the lower chamber being screwed down first, to avoid the compression of gases over the blood that would result if the upper clamp were fastened first. The tonometer was then raised

so that only the lower chamber remained in the bath, and the rubber tube was cut with scissors just below the upper clamp, leaving about 2 cm. of the tube projecting above the lower.

Into the upper, gas-containing chamber, about 50 cc. of mercury were run under pressure through the 3-way cock. The gas was thereby put under positive pressure, which facilitated drawing samples into the Haldane apparatus for analysis, and prevented the possibility of entrance of atmospheric air by leakage.

While the separated lower chamber still remained in the bath its 3-way cock was connected with a mercury leveling bulb. The clamp on the rubber outlet tube was then opened for a few seconds, while mercury was run into the chamber from below until the blood had risen into the rubber tube and displaced all the gas in the chamber. The clamp on the tube was then closed; the chamber was taken from the bath, clamped on a ring-stand, and removed for analysis of the blood.

Blood samples for analysis were drawn exactly as from the sampling tube described on page 131 (J, Fig. 3). In order to make the pipette tip fit tightly into the rubber outlet tube of the chamber, it was usually necessary to encircle the tip with a rubber ring cut from a section of tubing. This ring was lubricated with vaseline to facilitate fitting it into the rubber tube of the blood container.

Second Saturation Method. Final Tensions Estimated from Analyses of the Blood.—In the second method, used for larger volumes of blood than the first, a tonometer, modelled like a Barcroft gas sampling tube, with a single chamber (No. 2 of Fig. 3) of about 800 cc. capacity was employed. The volume of each tonometer, determined by weighing the water it held, was etched upon it. The gas mixture was made in the tonometer, as described in the first method, by admitting measured volumes of oxygen and CO_2, calculated by Equations 3 and 5 below. The measured volume of analyzed blood, usually about 75 cc., was drawn in through the lower cock after the significant gases had been introduced, and before the pressure was finally adjusted by admission of air, hydrogen, or nitrogen. In this case, the final total pressure at room temperature was kept 80 mm. below atmospheric by admitting the last gas through the mercury pressure regulator shown in Fig. 3. Otherwise, since the cocks were not opened

during saturation, they were likely to be forced out by the pressure which developed as the gases within warmed up in the bath. The tonometers were in all cases made of Pyrex glass.

When it was necessary to know exactly the final tension at equilibrium, but not necessary that this tension be precisely at a predetermined point, one saturation was sufficient. The initial gas mixture was prepared according to Equations 3 and 5 below, to give approximately a desired final tension, and the exact final tension was calculated after analysis of the saturated blood by Equation 4.

When, however, the plan of the experiment made it desirable to fix the final tension of CO_2 or O_2, or both, at exact, predetermined points, either two or three successive saturations were performed on the same blood in different tonometers. The tension for the first saturation was calculated according to Equation 3, while for the second and third, the gases were measured into the tonometers in such proportions as to produce the exact tension desired. The total number of saturations was two, if the tension change in the blood was slight, such as the change from 45 to 40 mm. of CO_2 tension; while three equilibrations were used if the change was larger, as when blood at 40 mm. was changed to 20 or 60. Analyses of the blood were performed after each of the last two saturations. Usually both results were identical. If there was a difference, the slight effect on the final tension was calculated by Equation 4.

Transfer of Blood from Tonometer to Tonometer in Second Saturation Method.—The receiving tonometer was evacuated, and the desired amounts of CO_2 and O_2 were run in, together with enough inert gas (H_2 or N_2) to make about half an atmosphere of pressure. The two tonometers were connected below by a capillary U-tube, the delivering tonometer having been wrapped in a towel wet with water at 38° as soon as it was removed from the bath. The upper cock of the delivering tonometer was opened and the connecting tube was filled with blood by manipulation of the lower cocks. Then all but a few drops of the blood was drawn over into the receiving tonometer. Sufficient inert gas was finally admitted to raise the pressure to $B - 80$ mm.

Transfer of Blood from Tonometer to Final Container in Second Saturation Method.—One 3-way cock of the tonometer was lifted

from the bath and connected with the mercury-filled receiving tube (J, Fig. 3), and the connections were filled with mercury. The tonometer was then inverted and placed upright in the bath, with the mercury-filled receiving vessel below and the upper end projecting from the bath. The upper cock was opened to the air, and the blood was drawn down into the receiving tube. In some instances the exchange was quickly performed outside the bath, the tonometer having been wrapped with a towel before removal from the water.

Calculations.

The formulas used in the calculations were developed as follows:

Let p_f = final tension in mm. of mercury, of a specified gas (CO_2 or O_2) in tonometer at end of saturation.

p_i = initial tension of the specified gas in tonometer at the beginning of saturation.

Δ = increase in total (free and combined) millimolecular concentration of the specified gas in the blood caused by changing the blood from its original state to that at the end of saturation. (Δ is negative if the concentration of the gas in the blood decreases during the saturation.)

T_{in} = absolute temperature of tonometer during saturation.

$T_{burette}$ = absolute temperature of burette from which gas is measured into tonometer.

V_{in} = cc. total volume content of tonometer.

$V_{burette}$ = cc. volume of the specified gas measured over water in the burette, at barometric pressure and $T_{burette}$, which must be transferred to the tonometer to give therein p_i tension of the gas at T_{in}.

V_{bl} = cc. volume of blood in tonometer during saturation.

V_{in}-V_{bl} = cc. volume of gas space in tonometer during saturation.

B = barometric pressure in mm. of mercury.

W = vapor tension of water in mm. of mercury.

If we place the volume of CO_2 or O_2 absorbed by the blood, equal to the volume lost by the gas phase to the blood during saturation, we obtain an equation from which may be calculated the initial tension necessary to secure a given final tension or the final tension resulting from a given initial one.

The number of cc. of CO_2 or O_2, reduced to 0°, 760 mm., absorbed by the blood during saturation is equal to the product of the total cc. of blood times the volume of gas absorbed by each

cc.   This product is $V_{bl} \times 0.0224\Delta$.   (The factor 0.0224 is the number of cc. of gas, reduced to 0°, 760 mm., contained in 1 cc. of a millimolecular solution of the gas.   Consequently 0.0224Δ is the volume of gas, reduced to 0°, 760 mm., absorbed by each cc. of blood.   The equations may be transformed into terms of volumes per cent of gas by substituting 0.01 for 0.0224.)

The total initial volume of the specified gas, reduced to 0°, 760 mm., in the gas space of the tonometer is $(V_{tn} - V_{bl}) \dfrac{p_i}{760} \dfrac{273}{T}$, and the final volume of the gas, at 0°, 760 mm., is $(V_{tn} - V_{bl}) \dfrac{p_f}{760} \dfrac{273}{T}$.   The volume of gas, at 0°, 760 mm., lost from the gas phase to the blood during saturation is the difference between the two, or $(V_{tn} - V_{bl}) \dfrac{p_i - p_f}{760} \dfrac{273}{T}$.   Placing the above two expressions equal to each other we have

$$0.0224 \, \Delta \, V_{bl} = \underbrace{(V_{tn} - V_{bl}) \frac{p_i - p_f}{760} \frac{273}{T}}$$

$$\underbrace{\phantom{0.0224 \, \Delta \, V_{bl}}}$$

Volume of
O_2 or CO_2
gained   by
blood.

Volume of O_2 or CO_2
lost by gas phase.   (1)

In order to find the CO_2 tension, p_i in the tonometer with which we must begin saturation to reach the desired final tension p_f we solve Equation 1 for p_i and obtain Equation 2.

$$p_i = p_f + \left(760 \times 0.0224 \Delta \frac{T_{tn}}{273} \frac{V_{bl}}{V_{tn} - V_{bl}} \right) \qquad (2)$$

When the numerical constants are combined this becomes

$$p_i = p_f + 0.0624 \frac{\Delta \, T_{tn}}{V_{tn} - V_{bl}} V_{bl}$$

$$= p_f + 19.4 \, \Delta \frac{V_{bl}}{V_{tn} - V_{bl}} \text{ when } T_{tn} = 311° = 38°C. \qquad (3)$$

Similarly if we fix the initial tension, p_i, and measure the change Δ in gas content of the blood, we can estimate the exact final tension of the specific gas at the end of equilibration as

$$p_f = p_i - 0.0624 \frac{\Delta T_{tn} V_{bl}}{V_{tn} - V_{bl}}$$

$$= p_i - 19.4 \Delta \frac{V_{bl}}{V_{tn} - V_{bl}} \text{ when } T_{tn} = 311° = 38°C. \tag{4}$$

For calculation of the volume $V_{burette}$ of gas which must be measured in the burette (Fig. 3) at atmospheric pressure and transferred to the tonometer to give therein the initial tension p_i, we have used Equation 5, which is developed by placing equal to each other the expressions indicating the volume of gas measured in the burette and in the tonometer, respectively, both being reduced to 0°, 760 mm. We then have

$$V_{burette} \times \frac{B\text{-}W}{760} \times \frac{273}{T_{burette}} = (V_{tn} - V_{bl}) \frac{p_i}{760} \frac{273}{T_{tn}}, \text{ whence}$$

$$V_{burette} = (V_{tn} - V_{bl}) \frac{p_i}{B\text{-}W_{burette}} \frac{T_{burette}}{T_{tn}}, \text{ or}$$

$$= \frac{p_i (V_{tn} - V_{bl})}{T_{tn}} \frac{T_{burette}}{B\text{-}W_{burette}} \tag{5}$$

Solving Equation 5 for p_i we obtain in Equation 6 the tension at bath temperature given by the gas volume, $V_{burette}$, measured into the tonometer.

$$p_i = V_{burette} \frac{T_{tn}}{V_{tn} - V_{bl}} \frac{B\text{-}W_{burette}}{T_{burette}} \tag{6}$$

We have found it convenient to use Equation 5 in the second of the two forms given above, for the reason that it places in one group the factors p_i, T_{tn}, and $(V_{tn} - V_{bl})$ which are independent of temporary room conditions and can therefore be calculated in advance, while the factors $T_{burette}$ and $(B - W_{burette})$, which must be determined at the moment of measurement, are placed together in a second group.

Approximate Estimation of Δ for Calculation of Initial Tensions of First Saturation.—This estimate was made by means of the average absorption curve of the kind of blood used. The manner in which the estimate was made is shown by an example. In the venous blood drawn from the horse used in most of our experiments the CO_2 tension was constantly in the neighborhood of 45 mm. The

average absorption curve of the animal's blood showed that changing the CO_2 tension produced approximately the following changes in CO_2 content:

P_f	CO_2 content.	Δ
mm	*mm.*	*mm.*
20	15.0	−6.6
40	20.5	−1.1
45	21.6	0.0
60	23.5	+1.9
80	25.2	+3.6

While the CO_2 content of the blood as drawn varied at times considerably from 21.6 mm., the values of Δ caused by given changes in tension remained fairly constant.

Exact Determination of Δ for Calculation of Final Gas Tensions.— This determination was made by comparison of the analyses of the blood after each of the last two saturations.

Example:

Equilibration No.	CO_2 content of blood.	Δ
	mm.	*mm.*
2	21.6	−0.4
3	21.2	

Limits of Error in Determination of Final CO_2 and O_2 Tensions. 1. *By Analysis of Gas Phase at End of Saturation (First Method).*— The analytical error of CO_2 and O_2 determinations by the Haldane apparatus is ordinarily about ± 0.02 volumes per cent. The tension corresponding to this at 760 mm. and 38° is $\frac{0.02}{100}$ (760 − 49) = 0.14 mm. of mercury. It appears probable that the error of estimating gas tensions produced by the "First saturation method" may be kept below 0.2 mm., since errors are practically excluded in separating the gas phase for analysis. As a matter of fact, results shortly to be published indicate that this degree of accuracy can be obtained quite consistently.

2. *By Analysis of Blood Before and After Saturation with Known Gas Mixtures (Second Method).*—The final tension is determined by Equation 4, which for 38° is

$$p_f = p_i + 19.4 \, \Delta \, \frac{V_{bl}}{V_{tn} - V_{bl}}$$

In determining the possible error in calculating p_f, we estimate and add the maximum errors in the experimental determinations of p_i and of the value $19.4 \, \Delta \, \frac{V_{bl}}{V_{tn} - V_{bl}}$, respectively, the latter value representing the correction which must be applied to p_i as the result of the loss or gain of significant gas by the blood during saturation.

p_i is determined by the amount of gas measured into the tonometer. From Equation 5 we have $p_i = \frac{V_{burette}}{V_{tn} - V_{bl}} \times$

$(B - W_{burette}) \times \frac{T_{burette}}{T_{tn}}$.

At the usual conditions, *viz.* $T_{burette} = 20° + 273$, $T_{tn} = 38° + 273$, $B = 760$, $B - W = 743$, $V_{tn} = 800$, $V_{bl} = 75$, we have $p_i = V_{burette} \times 1.17$. 1 cc. of gas, measured in the burette (Fig. 3), therefore corresponds to a tension of about 1.2 mm. of mercury in the tonometer. The gas can be measured with an error not exceeding 0.10 cc., so that p_i can be fixed within about $0.10 \times 1.2 = 0.12$ mm. by measurement. The errors in the other factors, *viz.* $(V_{tn} - V_{bl})$, $(B - W)$, and $\frac{T_{burette}}{T_{tn}}$ are relatively so small as to be negligible.

The error in determining the value of the term $19.4 \, \Delta \, \frac{V_{bl}}{V_{tn} - V_{bl}}$ may be estimated as follows: In our experiments the blood occupied about one-tenth as much space as the gases in the tonometer, $\frac{V_{bl}}{V_{tn} - V_{bl}} = 0.1$. Substituting 0.1 for the factor $\frac{V_{bl}}{V_{tn} - V_{bl}}$ in Equation 4, for 38°, we have $p_f = p_i - 1.94 \, \Delta$. That is, a change of 1 mm. in the value of Δ for CO_2 or O_2 causes a change of about 1.9 mm. in the tension of the gas at 38°. The analytical error of our blood gas determinations is about ± 0.05 mm.; and Δ represents the difference between two determinations, so that its possible error would be twice as great, or ± 0.1 mm.

The latter would cause an error of 1.9×0.1, or about 0.19 mm. in the calculation of the value, $1.94 \, \Delta \frac{V_{bl}}{V_{tn} - V_{bl}}$.

The total error in the determination of the final tension in the manner indicated by the equation $p_f = p_i + 19.4 \, \Delta \frac{V_{bl}}{V_{tn} - V_{bl}}$ we therefore estimate as approximately $0.12 + 0.19 = 0.3$ mm. of O_2 or CO_2 tension. This is of the same order of magnitude as the error involved in estimating the final tension by analysis of the tonometer gas, discussed above.

The error in the calculation from Δ may be reduced in either of two ways. (1) Since the error is chiefly due to the factors involved in the term $\Delta \frac{V_{bl}}{V_{tn} - V_{bl}}$, it may be reduced by reducing V_{bl}, the volume of blood used, and therefore the factor $\frac{V_{bl}}{V_{tn} - V_{bl}}$.

TABLE III.

Sample.	CO_2 tension calculated from volume of CO_2 measured into tonometer.	CO_2 tension calculated from analysis of tonometer gas.
	mm. Hg	*mm. Hg*
1	24.42	24.35
2	25.23	25.01

(2) When the final tension has been estimated by Equation 4, the blood may be saturated once more, at exactly this tension. The error is thereby practically reduced to that involved in fixing p_i, which is only 0.1 mm. of tension. This procedure has been followed when maximum accuracy has been required in the saturation of large volumes $(50 - 100$ cc.) of blood.

The following experiment indicates that with the apparatus used the two methods for fixing initial tensions agree within their limits of error:

Into two tonometers, in which the pressure had been reduced to about 60 mm., measured amounts of CO_2 were introduced from burette A (Fig. 3). Air was admitted until the pressure in the tonometers was brought to atmospheric. Samples of the gas mixture were then displaced from the tonometers into a Haldane air analysis apparatus and analyzed for CO_2. CO_2 tensions calculated from the volume of CO_2 introduced (Equation 5) and the tensions calculated from the results of the gas analyses, respectively, are shown in Table III.

TABLE IV.*

Formula No.	Use of formula.	Formula in terms of [H+] and K'.	Formula in terms of pH and pK'.
1	Calculation of [H₂CO₃] from p.	mm. $[H_2CO_3] = \dfrac{\alpha\,p}{760 \times 0.0221}$ $= 0.0587\,\alpha\,p$	
2	Calculation of [BHCO₃] from [CO₂] and p.	mm. $[BHCO_3] = [CO_2] - 0.0587\,\alpha\,p$	$pH = pK' + \log \dfrac{[CO_2] - 0.0587\,\alpha\,p}{0.0587\,\alpha\,p}$
3	Calculation of [H+] or pH from [CO₂] and p.	$[H^+] = K' \dfrac{[CO_2] - 0.0587\,\alpha\,p}{0.0587\,\alpha\,p}$	$pK' = pH - \log \dfrac{[CO_2] - 0.0587\,\alpha\,p}{0.0587\,\alpha\,p}$
4	Calculation of K' or pK' from [H+] or pH and [CO₂].	$K' = [H^+] \dfrac{[CO_2] - 0.0587\,\alpha\,p}{0.0587\,\alpha\,p}$	$p = \dfrac{[CO_2]}{0.0587\,\alpha\,(10^{pH - pK'} + 1)}$
5	Calculation of CO₂ tension from pH and [CO₂].	$p = \dfrac{[CO_2]}{0.0587\,\alpha\left(\dfrac{K'}{[H^+]} + 1\right)}$	
6	Calculation of [BHCO₃] from pH and [CO₂].	$[BHCO_3] = [CO_2] \dfrac{1}{1 + \dfrac{[H^+]}{K'}}$	$[BHCO_3] = [CO_2] \dfrac{1}{1 + 10^{pK' - pH}}$

* In order to use formulas with [CO₂], [BHCO₃], and [H₂CO₃], expressed in terms of volumes per cent of CO₂ instead of mm. concentration, replace the factor 0.0587 by the factor $\dfrac{100}{760}$, or 0.1316.

That the final tension can be set with a similar degree of accuracy is indicated by the constancy of the final CO_2 contents obtained in the accompanying second paper of the series. In the horse blood used a deviation of 0.7 volume per cent or 0.3 mM. in CO_2 content corresponds to a deviation of 1 mm. in CO_2 tension.

Formulas for Calculating pH, $[H^+]$, p_{CO_2}, $[BHCO_3]$, and $[H_2CO_3]$ from Data Usually Obtained by Direct Determination.

We have used certain rearrangements of Henderson's and Hasselbalch's equations so frequently that it appears desirable,

TABLE V.

Values of Constants for Formulas of Table IV.

	α	0.0587α	0.1316α	K'	pK'
Water................	0.555*	0.0326	0.0730	†	†
Serum or plasma........	0.541*	0.0318	0.0712	7.2×10^{-8}	6.14‡
Blood.................	0.511*	0.0300	0.0672	6.5×10^{-8}	6.18‡
12 per cent§ hemoglobin + 30 mM. NaHCO₃...	0.531‡	0.0312	0.0699	6.5×10^{-8}	6.18‡

* Bohr, 1905.
† Variable with NaHCO₃ concentration. See Hasselbalch, 1917.
‡ Data to be published. (K' and pK' determined on horse blood.)
§ Containing hemoglobin sufficient to bind 16 volumes per cent of oxygen. Millimolar hemoglobin concentration $= \dfrac{16}{2.24} = 7.15$ mM., assuming that 1 molecule of oxygen combines with 1 molecule of hemoglobin.

in order to avoid subsequent repetition, to record them here in connection with the description of technique.

The constants are given in terms of millimolecular (mM.) concentration, rather than in volumes per cent of gas, for the reason that comparison of concentration changes not only in oxygen and carbon dioxide, but also in electrolytes, such as chlorides and alkali protein compounds, has been necessary in the studies outlined, and a single unit of concentration that can be used throughout is desirable.

The following symbols are used:

$[CO_2]$, $[BHCO_3]$, $[H_2CO_3]$ = millimolecular concentration of total CO_2, $BHCO_3$, and H_2CO_3, respectively.

α = solubility coefficient of CO_2 at $38°$.

p = tension of CO_2 in millimeters of mercury.

K' = constant by which the ratio $\dfrac{[H_2CO_3]}{[BHCO_3]}$ must be multiplied to give $[H^+]$. Theoretically $K' = \dfrac{K}{\gamma}$ where K is the dissociation constant of H_2CO_3, γ the fraction of $BHCO_3$ dissociated into B^+ and HCO'_3 (Hasselbalch, 1917).

$pK' = -\log K'$ (Hasselbalch, 1917).

For the constants α, K', and pK', the values in Table V have been used.

In Formula 2 the $[BHCO_3]$ is calculated by subtracting from the total $[CO_2]$ the $[H_2CO_3]$, which is calculated from the CO_2 tension according to Formula 1.

Formula 3 is Henderson's familiar $[H^+] = K' \dfrac{[H_2CO_3]}{[BHCO_3]}$ with $[H_2CO_3]$ and $[BHCO_3]$ calculated according to Formulas 1 and 2.

Formula 4 is obtained by obvious rearrangement of Formula 3.

Formula 5 is obtained by solving Formula 3 or 4 for p.

Formula 6 is obtained by substituting the value of p from Formula 5 for p in Formula 2.

BIBLIOGRAPHY.

Adolph, E. F., and Ferry, R. M., *J. Biol. Chem.*, 1921, xlvii, 547.

Austin, J. H., and Van Slyke, D. D., *J. Biol. Chem.*, 1920, xli, 345.

Austin, J. H., and Van Slyke, D. D., *J. Biol. Chem.*, 1920–21, xlv, 461.

Barcroft, J., Bock, A. V., Hill, A. V., Parsons, T. R., Parsons, W., and Shoji, R., *J. Physiol.*, 1922, lvi, 157.

Bohr, C., *Skand. Arch. Physiol.*, 1905, *a*, xvii, 104.

Bohr, C., *Skand. Arch. Physiol.*, 1905, *b*, xvii, 205.

Christiansen, J., Douglas, C. G., and Haldane, J. S., *J. Physiol.*, 1914, xlviii, 244.

Doisy, E. A., Eaton, E. P., and Chouke, K. S., *J. Biol. Chem.*, 1922, liii, 61.

Evans, C. L., *J. Physiol.*, 1922, lvi, 146.

Fridericia, L. S., *J. Biol. Chem.*, 1920, xlii, 245.

Harrop, G. A., Jr., *Arch. Int. Med.*, 1919, xxiii, 745.

Hasselbalch, K. A., *Biochem. Z.*, 1917, lxxviii, 112.

Henderson, L. J., *Am. J. Physiol.*, 1908, xxi, 427.

Henderson, L. J., *J. Biol. Chem.*, 1920, xli, 401.

Henderson, L. J., *J. Biol. Chem.*, 1921, xlvi, 411.

Henderson, Y., *J. Biol. Chem.*, 1918, xxxiii, 31.

Hill, A. V., *J. Biol. Chem.*, 1922, li, 359.

McLean, F. C., Murray, H. A., Jr., and Henderson, L. J., *Proc. Soc. Exp. Biol. and Med.*, 1919–20, xvii, 180.

Parsons, T. R., *J. Physiol.*, 1919–20, liii, 42.

Peters, J. P., Jr., Barr, D. P., and Rule, F. D., *J. Biol. Chem.*, 1920–21, xlv, 489.

Van Slyke, D. D., *J. Biol. Chem.*, 1917, xxx, 347.

Van Slyke, D. D., *Proc. Nat. Acad. Sc.*, 1921, *a*, vii, 229.

Van Slyke, D. D., *Physiol. Rev.*, 1921, *b*, i, 141.

Van Slyke, D. D., *J. Biol. Chem.*, 1922, lii, 525.

Van Slyke, D. D., and Cullen, G. E., *J. Biol. Chem.*, 1917, xxx, 289.

Van Slyke, D. D., and Stadie, W. C., *J. Biol. Chem.*, 1921, xlix, 1.

STUDIES OF GAS AND ELECTROLYTE EQUILIBRIA IN BLOOD.

II. THE REVERSIBILITY OF THE EFFECTS OF CHANGES IN CO_2 AND O_2 TENSIONS ON THE CO_2 CONTENT OF DEFIBRINATED HORSE BLOOD.

By JOHN P. PETERS, GLENN E. CULLEN, AND J. HAROLD AUSTIN.

(From the Hospital of The Rockefeller Institute for Medical Research.)

Haggard and Henderson[1] have stated that when the CO_2 tension of dog blood, either defibrinated or oxalated, has been reduced to less than about 20 mm. the blood undergoes an irreversible, or at least not readily reversible, change characterized by a diminution of its CO_2 capacity. When this blood was again brought into equilibrium with a gas mixture with a higher CO_2 tension it took up less CO_2 than before its exposure to the low CO_2 tension. They found this irreversible change more consistently in defibrinated than in oxalated blood.

Reversibility of the Effect of Low CO_2 Tension.—In order to determine whether such an irreversible change occurred in defibrinated horse blood the following experiments were carried out. Fresh horse blood was defibrinated by stirring under oil and was then strained through gauze under oil. The blood was divided into two portions. One of these was first exposed to a low tension of CO_2 and then saturated at 60 mm. CO_2 tension. The other was at once saturated at 60 mm. CO_2 tension. The CO_2 contents of the two were then compared. The "second saturation method" described in the first paper of these studies was used.[2] The blood gases were determined by the blood gas apparatus described by Van Slyke and Stadie,[3] as the constant volume apparatus was not yet in use.

[1] Haggard, H. W., and Henderson, Y., *J. Biol. Chem.*, 1920–21, xlv, 209.

[2] Austin, J. H., Cullen, G. E., Hastings, A. B., McLean, F. C., Peters, J. P., and Van Slyke, D. D., *J. Biol. Chem.*, 1922, liv, 121.

[3] Van Slyke, D. D., and Stadie, W. C., *J. Biol. Chem.*, 1921, xlix, 1.

The first portion was exposed at 38° in a tonometer which had been filled with air free from CO_2. The final CO_2 tension in this tonometer, due to CO_2 evolved from the blood, was calculated to be 15 ± 2 mm. This tension was further confirmed by the location of the CO_2 content of the blood from this tonometer on the CO_2 absorption curve of the blood. The blood was then passed through a series of three tonometers at 38°. Into the first was introduced enough CO_2 to produce a CO_2 tension of 60 mm. at 38° after allowance had been made for the CO_2 which would be taken up by the blood. Into the two succeeding tonometers was introduced the exact amount of CO_2 required to give a tension of 60 mm., assuming no further removal of CO_2 by the blood. The blood was analyzed for CO_2 after exposure in each of the last two tonometers.

The second portion of blood was also passed through a series of four tonometers at 38° for the same periods of time. Into each of these tonometers was introduced the amount of CO_2 required to give a tension of 60 mm. at 38°. (The CO_2 placed in the first tonometer was corrected for the CO_2 which it was estimated the blood would absorb.) The blood from the last two tonometers was analyzed for CO_2.

The above experiment was duplicated on a different sample of blood from the same horse. The results of the two experiments are given in Tables I and II.

Reversibility of the Effect of Low Oxygen Tension.—Blood was withdrawn under albolene from the jugular vein of a horse, defibrinated by gentle stirring with a glass rod, and filtered through gauze, under a layer of albolene. It was divided into two portions (Nos. 1 and 2) of 50 cc. each. One of these (No. 2) was exposed in a tonometer at 38°C. to 40 mm. of CO_2 in commercial nitrogen (No. 2 a), and then transferred to a second tonometer and exposed to a mixture of 40 mm. of CO_2 and 13 mm. of oxygen in commercial nitrogen. A sample was then removed and analyzed for CO_2 and oxygen (No. 2 b). The remainder was transferred successively to three more tonometers (Nos. 2 c, 2 d, and 2 e) in each of which it was exposed to 40 mm. of CO_2 in air. (In making up the gas mixtures in tonometers 2 a and 2 c an allowance was made for the change of CO_2 and oxygen content in the blood.) Samples were removed from Nos. 2 d and 2 e

and analyzed for CO_2. The amount of CO_2 in both was identical, demonstrating that complete gaseous equilibrium had been established.

TABLE I.

Saturation.	Exposed to low CO_2 tension.		Not exposed to low CO_2 tension.	
	CO_2 tension.	CO_2 content.	CO_2 tension.	CO_2 content.
	mm.	*vol. per cent*	*mm.*	*vol. per cent*
First.................	15*	33.0	60*	
Second...............	60*		60	
Third.................	60	53.7	60	54.4
Fourth...............	60	53.7	60	54.45

* Approximate.

TABLE II.

Saturation.	Exposed to low CO_2 tension.		Not exposed to low CO_2 tension.	
	CO_2 tension.	CO_2 content.	CO_2 tension.	CO_2 content.
	mm.	*vol. per cent*	*mm.*	*vol. per cent*
First.................	15*		60*	
Second...............	60*		60	
Third.................	60	53.1	60	53.8
Fourth...............	60	53.8	60	53.5

* Approximate.

TABLE III.

No	Treatment of blood.	CO_2 content.	O_2 content.
		vol. per cent	*vol. per cent*
1 e	Exposed in four successive tonometers to 40 mm. of CO_2 in air.	48.3	14.8
2 b	Exposed in two successive tonometers to 40 mm. of CO_2 and 13 mm. of O_2.	51.7	5.1
2 e	Same blood exposed in two more successive tonometers to 40 mm. of CO_2 in air.	48.1	14.8

Meanwhile the other specimen (No. 1) had been passed through the same number of tonometers, in each of which it had been exposed to 40 mm. of CO_2 in air. Analysis of the last specimen (No. 1 e) for CO_2 and O_2 gave results identical with those obtained

from specimen No. 2 e, which had first been exposed to CO_2 in nitrogen. Similar results have been obtained in other experiments.

It is evident from these experiments that the reduction of the CO_2 tension of defibrinated horse blood to as little as 15 mm. produces no irreversible change in the carbon dioxide capacity of the blood. There appears to be no doubt of the reversibility of the reactions between 15 and 60 mm. of CO_2 tension. It seems likely, as Evans[4] suggests, that the irreversible fall in CO_2 capacity, observed by Haggard and Henderson[1] was due to the formation of acid in the blood. This acid formation is very rapid in dog blood[2] and is accelerated, according to Evans, by reduction of CO_2 tension (increase of pH).

Our results further indicate that the effect of reduced oxygen tension on the CO_2 capacity of blood is entirely reversible.

[4] Evans, C. L., *J. Physiol.*, 1922, lvi, 146.

A SYSTEM OF BLOOD ANALYSIS.

A REVISION OF THE METHOD FOR DETERMINING URIC ACID.

By OTTO FOLIN.

(From the Biochemical Laboratory of Harvard Medical School, Boston.)

Protein Precipitation versus Loss of Uric Acid.

In two recent papers Pucher[1] reports that only 75 per cent of the uric acid added to sheep blood is recovered in the tungstic acid blood filtrate prepared according to the directions of Folin and Wu. By using an alternative process, described but not recommended by Folin and Wu, Pucher's recovery of uric acid rose to 93 per cent. The alternative process consists of heating the blood paste, obtained in the regular process of precipitation, in boiling water for a few minutes before filtering.

It is scarcely necessary to go into a full discussion of the objections to the heat process for securing protein-free blood filtrates; for all will doubtless agree that if it is not essential it is better not to use it. Neither creatinine nor the amino-acid nitrogen can be correctly determined in heated blood filtrates. Such filtrates also contain more of the material (other than uric acid) capable of yielding a color with the uric acid reagent.

In the original paper on the tungstic acid method for precipitating the blood proteins it is stated that 10 or even 20 mg. of added uric acid are quantitatively recovered from the blood filtrates obtained in that process. That statement was based on careful check work made by Dr. Wu and myself, both together and separately. I had, therefore, little reason to believe that the losses reported by Pucher could represent anything but some unsuitable condition present in his experiments. The sodium

---

[1] Pucher, G. W., *J. Biol. Chem.*, 1922, lii, 317, 329.

tungstate which he used might not have been the true ortho-tungstate, or he might not have used proper precautions in the final step, the addition of the sulfuric acid. Pucher states, to be sure, that he carefully followed the directions given by Folin and Wu in the making of his precipitations. But from the description given in his second paper (p. 332) it would appear that he has paid little or no attention to one important point; namely, the shaking of the diluted blood tungstate mixture while adding the sulfuric acid. The subsequent vigorous shaking, which he emphasizes, cannot liberate adsorbed or occluded uric acid. I must admit in the light of my present experience that Folin and Wu did not ade-quately emphasize the importance of constant shaking while the sulfuric acid is added; but it is obvious that unless this is attended to, local zones of excessive acidity will be produced. Such temporary excess of acid, particularly if the tungstate is not the best, may be more disastrous than is the addition of too much acid at the finish.

Notwithstanding my certainty that added uric acid had been quantitatively recovered by Wu and myself, it seemed best to repeat the work. In this check work I have encountered some surprising results. Different samples of sheep blood exhibit great differences in their tendency to retain added uric acid in the precipitated proteins. The same blood when kept 5 to 6 days in a cold room (4°C.) does not remain stationary in its tendency to hold back added uric acid. It will usually lose this tendency on standing, but in at least one sample of sheep blood from which I recovered 97 per cent when fresh I could get only 92 per cent re-covery 5 days later. I have examined twenty-two different samples of sheep blood, and three samples of calf blood. From each of the latter I recovered 93 per cent, when 10 mg. were added.

On the basis of the numerous experiments which I have now made I am no longer prepared to say that 95 to 100 per cent recovery of added uric acid is always attainable. Our earlier findings on this point must be due to the fact that we happened to deal with samples of blood that did not possess any great tendency to retain uric acid. The behavior of different samples of sheep blood with respect to their ability to retain uric acid is quite extraordinary. With some samples there is absolutely no loss, while with others I have not been able so to make the precipitation

at the ordinary 1 in 10 dilution as to be sure of more than 90 per cent recovery. The heat process recommended by Pucher is also quite inadequate; with some bloods it gives lower values than are obtained without heating. The maximum recovery (from 90 to 100 per cent, depending on the sample of blood), is obtained as follows:

After the blood has been diluted with 7 volumes of water and 1 volume of 10 per cent sodium tungstate, add very slowly (drop by drop) with constant shaking four-fifths of the required $\frac{2}{3}$ N sulfuric acid—that is 4 cc. for 5 cc. of blood, or 8 cc. for 10 cc. of blood—and let the mixture stand for 20 to 30 minutes before adding the remaining one-fifth of the sulfuric acid. The last portion of acid must also be added drop by drop with continuous shaking.

The extra loss which may be encountered by adding all the required sulfuric acid at once, if the addition is made slowly and with shaking, is only about 3 per cent.

That losses of 8 to 10 per cent of uric acid *may* occur during the precipitation of the proteins from whole blood must be accepted for the present. I must have made at least 200 experiments trying to recover the last 8 to 10 per cent of the added 10 mg. of uric acid from bloods which showed a strong tendency to retain it. The problem is intrinsically interesting, because different bloods behave so differently. Practically it is of comparatively little importance. When very much uric acid is present, as in nephritic retentions, it is advantageous to make the protein precipitation at a dilution of 1 in 20, instead of the customary 1 in 10, and at this higher degree of dilution the uric acid, up to at least 20 mg., is quantitatively recovered. When working with plasma there is no retention or loss of uric acid.

For the investigation of the extent to which uric acid added to sheep blood goes into the filtrate I have used the direct determination described on page 167. All possibility of error due to unknown constituents of the blood was eliminated by adding to the standard uric acid solution the same quantity of the sheep blood filtrate as was used for the determination. The filtrate added to the standard was prepared from the same blood, but contained no added uric acid.

The Preparation of Standard Uric Acid Solutions.

The determination of uric acid described[2] in the "System of blood analysis" was adapted to the use of a new standard solution of uric acid—the sulfite standard. It was thought that the finding of this very stable standard would more than make up for the fact that the intensifying effects of the cyanide on the color obtained had to be sacrificed. I have had several lots of the sulfite standard keep for more than 2 years, although reports received from different laboratories show that such keeping quality is by no means always obtained. Results reported by Pucher indicate that bacterial decompositon may come in, a possibility which we had omitted to provide for, because it was thought that 10 per cent sodium sulfite would prove an adequate preservative. The addition of preservatives, such as chloroform or toluene, to standard solutions is not entirely satisfactory, because pipettes never run clean with such solutions. It is not worth while to discuss how to insure the keeping quality of the sulfite standard because from recent publications (Morris, Palmer, Benedict) it is clear that many workers will not forego the advantages of the cyanide for the sake of a permanent uric acid standard. To clinical laboratories the keeping quality of standard solutions is of much greater importance than the investigators mentioned seem to realize, and if I in this paper discard the sulfite standard, it is only because I believe that I have found another, even more dependable solution of uric acid, which is free from the objections raised against the sulfite solution.

The standard solution of uric acid described below is not new; it was used in this laboratory 10 years ago and was described in 1913.[3] The uric acid-preserving factor is formaldehyde. Formaldehyde forms very soluble addition products with uric acid. These addition products are not decomposed when the solutions are acidified. I once kept such a solution on a warm radiator for 3 months and could detect no change. No other kind of uric acid solution could withstand such treatment, and it was thought that a really permanent standard solution of uric acid had been found. At a later period when I started to use the

[2] Folin, O., and Wu, H., J. Biol. Chem., 1919, xxxviii, 100.

[3] Folin, O., and Denis, W., J. Biol. Chem., 1913, xiv, 96.

standard it was nevertheless found to have changed. It gave more color than it did when originally standardized.

At that time we were more interested in other problems, and the cause of the change in the uric acid solutions was never investigated; the standard was simply abandoned. In making use of these formaldehyde solutions of uric acid we had adapted the formaldehyde standard to the uric acid method, as it was then employed, and neglected to investigate the behavior of the standard when diluted with water. From 5 mg. of uric acid we obtained only as much color as is given by 1 mg. of free uric acid, and accurate results were obtained only because the reaction was always made in exactly the same way. In 1915 the formaldehyde standard was investigated and condemned by S. R. Benedict.[4] Like ourselves, Benedict found that the formaldehyde-uric acid solutions suffered no deterioration on standing, but he also found that the color obtained was too uncertain to permit its use as a standard. Benedict, like ourselves, omitted to study the formaldehyde-uric acid solutions in any great detail and the intrinsic merits of these solutions have thus remained undiscovered.

Since the uric acid-formaldehyde combinations dissociate into their component parts on diluting with water, it occurred to me that the solutions had never been used to the best advantage. If it should be feasible by suitable dilution to accomplish the complete dissociation of the formaldehyde-uric acid compounds so that all of the uric acid present would react with the uric acid reagent, then we should have removed the sources of uncertainty attached to the solutions as originally employed; yet would retain the remarkable keeping quality accomplished by means of formaldehyde. The intensifying effects of the cyanide would be retained. Formaldehyde, unlike the sulfite, does not counteract the "driving power" of the cyanide.

It was soon found that the degree of dilution required to set free all the uric acid comes well within the dilutions now used for standard uric acid solutions in connection with uric acid determinations in urine, and for uric acid determinations in blood the standard solutions are still weaker, so that this aspect of the problem was easily solved. Uric acid can, however, form several addition products with formaldehyde. Some of the addition

[4] Benedict, S. R., and Hitchcock, E. H., *J. Biol. Chem.*, 1915, xx, 621.

products may not be so readily dissociated by dilution with water, and the possibility remained that on very long standing (for months or years) less readily dissociated compounds might be formed. The speed and the extent to which such compounds may be formed will necessarily depend on the concentration of the formaldehyde present in the solutions. For practical purposes it is therefore necessary to find out the concentrations of formaldehyde which are best suited for the preparation of the standard uric acid solutions. The solvent effects of formaldehyde on uric acid are exceedingly strong; by the aid of heat a 40 per cent solution of uric acid can be prepared with the help of ordinary undiluted (40 per cent) formaldehyde. The germicidal properties of formaldehyde are also very pronounced. 1 part of 40 per cent formaldehyde in 1,000 parts of bouillon is said to prevent the development of putrefactive bacteria. There should, therefore, be no difficulty about preparing stock solutions of uric acid so weak in formaldehyde that no unduly stable compounds with the formaldehyde are produced on long standing.

On the other hand, from the standpoint of possible *chemical* decomposition of the uric acid, it should be advantageous to use the largest permissible excess of formaldehyde, because in as far as there is chemical decomposition of uric acid this will occur at the expense of the dissociated material, and the greater the concentration of formaldehyde the less free, or reactive uric acid will be present.

On the 1st of March I prepared a series of solutions containing 25, 50, 100, and 200 cc., respectively, of 40 per cent formaldehyde. All contained 1 mg. of uric acid per cubic centimeter except the last, which contained 2 mg. per cubic centimeter. The solutions were kept in the dark and in small tightly corked bottles. At the end of 3 months 10 cc. samples from each lot were diluted to a uric acid concentration of 1 mg. per 10 cc. and the reactive uric acid was immediately determined by comparison with a freshly prepared solution of uric acid. The following colorimetric readings were obtained:

		cc. 25	cc. 50	cc. 100	cc. 200
40 per cent formaldehyde per liter.	Standard.	25	50	100	200
Colorimetric reading............	20	20	20.5	21.7	22.5

The solutions were then further diluted to a concentration of 1 mg. of uric acid for 250 cc. (the concentration used in blood analysis) and the colorimetric determinations were made immediately according to the process described on page 167. The colorimetric readings obtained were nearly identical with those recorded above. 45 minutes later the determinations were repeated. This time all the dilute solutions gave the same color value as the standard.

Similar series of determinations were made at the end of 4 months. The values obtained were substantially identical with the corresponding values obtained a month before. From these data it seems safe to draw the following conclusions:

1. No permanently undissociable compounds are formed between uric acid and formaldehyde.

2. In the presence of more than 50 cc. of 40 per cent formaldehyde per liter, appreciable amounts of addition products between uric acid and formaldehyde are formed—products which are not completely dissociated when the solutions are diluted ten times, that is, to the uric acid concentration represented in the standard uric acid solutions usually employed in urine analysis.

3. At the much greater dilution corresponding to that of the standard in blood analysis all the uric acid-formaldehyde compounds are completely dissociated within 45 minutes.

4. The uric acid remains quantitatively intact in formaldehyde solutions.

5. Uric acid solutions made with the help of 25 to 50 cc. of formaldehyde per liter are all serviceable for standard solutions.

The solution described by Folin and Denis in 1913 contained 40 cc. of formaldehyde per liter. On the whole it is, perhaps, safest to recommend the use of 25 cc. of formaldehyde since there appears to be no danger of either chemical or bacterial decomposition in such solutions; but it is obvious that there is room for very wide variations, not only with reference to the formaldehyde content but also in the acid content of these solutions.

The directions given below are made specific only in the interest of those who have not the time to try out other variations.

Transfer exactly 1 gm. of uric acid to a funnel on a 300 cc. flask. Transfer from 0.45 to 0.5 gm. of lithium carbonate to a 300 cc. beaker, add 150 cc. of water, and heat to 60° C., shaking or stirring until all the carbonate has dissolved. With the hot

carbonate solution rinse the uric acid into its flask and shake. The uric acid dissolves practically at once. As soon as a clear solution is obtained, cool under running water, with shaking, and transfer to a volumetric liter flask. Rinse and dilute to a volume of 400 to 500 cc. Add 25 cc. of 40 per cent formaldehyde, and after shaking to insure thorough mixing, acidify by the addition of 3 cc. of glacial acetic acid, or equivalent quantities of 50 or 25 per cent acetic acid. Shake, to remove most of the carbonic acid, dilute to volume, and mix. Fill up to the neck a series of small bottles (100 to 150 cc.) with the stock solution, cork very tightly, label, and keep in a dark place.

For actual use the standard stock solution containing 1 mg. per cc. is diluted 250 times. For urine analysis a suitable dilution is 5 cc. in 50 cc., having 1 mg. of uric acid per 10 cc. of solution.

The keeping quality of the diluted standards is a matter of minor importance, but these solutions should keep at least as long as any other solutions having the same concentration of uric acid. Statements as to how long such solutions will keep cannot be very dependable. The keeping quality will depend partly on the temperature, partly on the degree of cleanliness. In clinical laboratories solutions are more apt to deteriorate because of excessive bacterial infections. The dilute standard described on page 165 will keep a long time.

Colorimetric Uric Acid Determinations.

The uric acid reagent of Folin and Denis was described in 1912 and has been used ever since practically without modification and by practically all who have made uric acid determinations. The reagent is also used in other determinations, such as for the assay of adrenalin solutions, and it has proved very serviceable for the colorimetric estimation of cystine. In 1920 the reagent was subjected to a thorough examination; its active ingredients were isolated and identified by Wu[5] in the course of his excellent survey of the phosphotungstic and phosphomolybdic acids.

Wu's stimulating paper has been followed by a series of attempts to prepare other uric acid reagents. A short time ago there appeared, almost simultaneously, descriptions of no less than

[5] Wu, H., *J. Biol. Chem.*, 1920, xliii, 189.

four new and different uric acid reagents—all recommended by
their authors as better, in one respect or another, than the uric
acid reagent of Folin and Denis. One of these new reagents, that
of Jackson and Palmer,[6] may be described as representing an
effort to purify, by dialysis, the original reagent of Folin and Denis.
In two of the new reagents, that of Morris,[7] and one of Benedict's,[8]
arsenic acid is substituted for phosphoric acid, and in the fourth
(Benedict's) a part only of the phosphate is replaced by arsenic.

There are, now therefore, five different uric acid reagents,
whereas during the past 10 year period all used the same one.
Nor is there any reason to believe that the list has been exhausted,
because many other analogous mixtures can undoubtedly be
prepared. (See, for example, the mixture referred to on page 163.)
Whatever may be the final outcome of such a multiplicity of similar
reagents for one specific purpose, the immediate effect will
necessarily be some uncertainty and confusion—particularly
among those interested only in the clinical applications of the
methods for blood analysis. Without entering upon a discussion
of the relative merits of the different new uric acid reagents, I
believe that it is well within the truth to say that uric acid in blood
can be determined as accurately and as conveniently by means of
the uric acid reagent of Folin and Denis as by any other reagent
yet described.

Some reference must necessarily be made to the mixed arsenic-
tungsten reagent of Benedict, because this mixture is said to
possess a special selective reaction with uric acid, a selective
reaction not obtainable with the uric acid reagent of Folin and
Denis. Because of the extraordinary "specificity" of this
reagent, Benedict is now able to obtain trustworthy uric acid
values by applying the color reaction directly to blood filtrates
without any preliminary isolation of the uric acid.

If Benedict's findings and interpretations were correct, this new
uric acid reagent would naturally and inevitably take the place
formerly held by the reagent of Folin and Denis. Benedict's
technique involves other new conditions besides the use of his uric
acid reagent. Benedict, Morris, and Palmer have all, seemingly

[6] Jackson, H., Jr., and Palmer, W. W., *J. Biol. Chem.*, 1922, l, 90.

[7] Morris, J. L., and Macleod, A. G., *J. Biol. Chem.*, 1922, l, 60.

[8] Benedict, S. R., *J. Biol. Chem.*, 1922, li, 189, 190.

independently, discovered the interesting fact that by omitting
the addition of any other alkali than sodium cyanide the intensity
of the color obtained from a given quantity of uric acid is very
much increased, above that obtained with cyanide plus carbonate.
Benedict has made the additional important discovery that by
the application of heat (water bath) the color obtained is again
greatly intensified. By the combination of the right amount of
cyanide as the only alkali, and heat, Benedict has obtained so
much color that 5 cc. of blood filtrate, corresponding to 0.5 cc. of
normal blood, give all the color needed for an exact colorimetric
comparison.

The question raised in my mind was whether the results re-
ported by Benedict were not to be explained on the basis of these
new conditions rather than on the use of a more suitable uric acid
reagent. This conjecture proved correct. The reagent of Folin
and Denis can be made to yield a color with blood filtrates which
corresponds very closely to their true uric acid content. To obtain
such remarkable results one needs only to apply, with certain
variations, the favorable conditions discovered by Benedict.

I am not prepared to agree with Benedict that such excellent
results are due wholly to the specificity of the uric acid reagents.
On the contrary, I am quite sure that correct results are obtained
only by so adjusting the conditions that two sets of errors balance
each other. For practical purposes, however, the explanation is
immaterial so long as dependable results are obtained.

In order to illustrate the great specificity of his new uric acid
reagent Benedict cites experiments with resorcinol. This poly-
phenol gives very much less color than does an equal amount of
uric acid, and when the reaction is made on mixtures of resorcinol
and uric acid, the color obtained is so much less than the sum of
what the two would give separately that several times as much
resorcinol as uric acid can be present before there is any material
increase in color above that given by the uric acid alone. Bene-
dict has evidently not made any analogous experiments with the
uric acid reagent of Folin and Denis, for if he had done so he
could not have failed to find that his reagent gives several times as
much color with resorcinol as is obtained with the reagent of
Folin and Denis. When applied to mixtures of uric acid and
resorcinol the latter reagent produces less color than is obtained

from the uric acid alone. In the presence of five times as much resorcinol as uric acid the loss in color amounts to about 10 per cent.

The effect of resorcinol on the uric acid reaction is evidently opposite to that produced by cyanide. This effect is of the same order with the two uric acid reagents, but in the case of Benedict's reagent the loss in color from the uric acid is more than counter-balanced by the color obtained from the resorcinol and the total color is therefore greater than that given by the uric acid alone.

By using suitable mixtures of the two reagents (3 volumes of the Folin-Denis reagent to 1 volume of Benedict's) the two effects of resorcinol can be made to balance each other exactly. With 0.5 cc. of this mixture plus 1 cc. of 15 per cent sodium cyanide, resorcinol, up to ten times the amount of uric acid present, has no effect on the color produced.

While blood filtrates undoubtedly contain traces of resorcinol they also contain many other products capable of interfering with the reaction by which the uric acid is determined. While working with the filtrates from sheep blood I examined this aspect of the uric acid determination. Sheep blood usually contains less than 0.1 mg. of uric acid per 100 cc. Sheep bloods also contain large and very variable amounts of products capable of interfering with the uric acid reaction. If a truly specific uric acid reagent were available it should be capable of giving the correct value for the uric acid added directly to sheep blood filtrates. At one period it seemed as though the uric acid reagent of Folin and Denis were equal to this situation. Very satisfactory results were obtained with two or three samples of sheep blood filtrate. Later, however, the elusive character of the results became clear, for when other sheep blood filtrates were tried (positive) errors up to 20 or even 30 per cent were found. Nor did Benedict's reagent give better results. If the direct determination of uric acid in filtrates from human blood, whether by Benedict's reagent or by the reagent of Folin and Denis, gives dependable results, it is only because these filtrates contain much less of the interfering materials than do sheep blood filtrates. This being the case, it is, I think, quite necessary to retain at least for check purposes, the preliminary isolation process outlined by Folin and Wu. In the course of my efforts to determine directly the uric acid added to sheep blood filtrates some interesting and valuable information was gained.

It was found that to get the maximum color from a given quantity of uric acid and the widest range of proportionality for different amounts, it is necessary to increase the amounts of uric acid reagent and of cyanide up to a point where it is very difficult to avoid the formation of precipitates. Benedict encountered the same difficulty, and to meet it recommends that sodium oxalate be substituted for potassium oxalate to prevent the clotting of blood. Sodium oxalate dissolves very slowly in either cold or hot water. It dissolves only to the extent of about 3 per cent. Sodium oxalate is, therefore, not very suitable. Lithium oxalate is better. This salt has about twice the solubility of sodium oxalate.

Lithium oxalate is easily prepared: To 50 gm. of lithium carbonate in a 2 liter beaker add 85 gm. of oxalic acid. To the mixture add 1 liter of hot water. Stir cautiously. Complete solution is obtained practically as soon as the carbonic acid has passed off. Evaporate to dryness and powder. 1 mg. of lithium oxalate is equivalent to 2 mg. of potassium oxalate, and 1 mg. of lithium oxalate per cc. of blood is, therefore, quite enough for the prevention of clotting.

Potassium salts are by far the most serious cause of precipitate formation in the colorimetric uric acid determination. But sodium salts, particularly in the form of cyanide, are also very apt to produce precipitates when present in too large amounts. Lithium salts differ from both. Lithium salts, in excess, tend to produce precipitates at higher temperatures, while at room temperatures they have the opposite effect. They actually prevent the formation of sodium or potassium precipitates at ordinary room temperatures. I, therefore, recommend the use of lithium oxalate to prevent the clotting of blood and the use of some other lithium salt (sulfate, chloride, or citrate) as an additional reagent in the making of colorimetric uric acid determinations.

While an excess of lithium oxalate does no harm in connection with the uric acid determination, the indiscriminate addition of any oxalate (or citrate) to blood to prevent clotting is undesirable, because a large excess interferes with the protein precipitation and also complicates the urea determination. When much oxalate is present the blood filtrate must have an excess of acid added, and

this extra acid stays in the filtrate in the form of free oxalic acid. Lithium oxalate (or potassium oxalate) for the prevention of blood clotting is most conveniently used in the form of oxalated paper or cloth. The cloth is better than the paper because the latter is apt to break up into fibers which clog the blood pipette.

To prepare oxalated cloth proceed as follows: Cut up about 75 to 80 gm. of bird's eye cotton cloth, free from starch, into strips about 10 cm. wide and 40 to 50 cm. long. Transfer 10 gm. of lithium carbonate and 17 gm. of oxalic acid to a liter beaker. Add 240 cc. of hot water (75° C.). Complete solution is obtained in a few seconds. Transfer the warm solution to a plate; pass the cotton strips through it, and hang up on a string to dry—exactly as in the making of litmus paper. The cloth, when dry, will carry about 20 per cent of lithium oxalate. 50 mg. of such cloth will easily prevent the clotting of 15 or even 20 cc. of blood. If desired, the oxalate content can be doubled by repeating the process.

The direct determination of uric acid in the blood filtrates from the tungstic acid precipitation described below will be found to give results which I believe to be dependable when applied to human blood. The values obtained are usually 0.1 or 0.2 mg. above those obtained by the silver precipitation and sodium chloride extraction.

Solutions.

1. Diluted uric acid standard containing 0.02 mg. of uric acid in 5 cc.

From the standard uric acid-formaldehyde solution, containing 1 mg. per cc., transfer, with an Ostwald pipette, 1 cc. to a 250 cc. volumetric flask. Half fill the flask with water, and add 10 cc. of the $\frac{2}{3}$ N sulfuric acid used in blood protein precipitation. Add also 1 cc. of 40 per cent formaldehyde (*but no more*) and then dilute to volume, mix, and date.

I hesitate about making any definite statement as to how long these diluted solutions will keep. In the course of 5 weeks of occasional use of one such solution I have found no deterioration whatever.

2. Uric acid reagent of Folin and Denis.

3. Lithium sulfate solution. Dissolve 20 gm. of powdered lithium sulfate (Baker and Adamson's) in about 80 cc. of cold

water. Dilute to a volume of 100 cc. and filter from the slight amount of insoluble matter present.

4. Approximately 15 per cent solution of sodium cyanide in 0.1 N sodium hydroxide.

Cyanide solutions may be said to improve with age, and it is therefore advisable to prepare enough for at least 3 months use. The solid sodium cyanide should be white. Old weathered samples of cyanide which have been decomposed by the carbon dioxide of air cannot be used. Weigh out on a paper from 100 to 450 gm. of the cyanide. Transfer to a large beaker, add 6.7 cc. of 0.1 N sodium hydroxide for each gram of cyanide taken, stir occasionally until the whole has dissolved. The solution is opalescent due to the presence of a little insoluble matter. Transfer to a bottle, label, and let stand for 2 weeks or longer before using.

Some explanations are necessary concerning the preparation and behavior of sodium cyanide solutions.

Cyanide solutions turn yellow in the course of time (2 to 3 weeks). This discoloration is faster in concentrated than in dilute solutions. It is prevented by free exposure to the air. Aqueous cyanide solutions kept in beakers covered with watch-glasses remain colorless. The formation of the brown substance ("azulonic acid") is also prevented by the addition of a little alkali. This decomposition, with discoloration, of the cyanide solution is of little or no importance in connection with uric acid determinations, but it seems best to prevent it since its prevention is so easily accomplished. The use of 0.1 N, or even 0.05 N, sodium hydroxide instead of water furnishes ample protection against this decomposition.

The important point to be noted about cyanide solutions is not the decomposition products formed on standing, so much as the presence of impurities in the original material. Practically all freshly prepared cyanide solutions contain notable quantities of reducing substances, which give a blue color with the uric acid reagent. Different brands of sodium cyanide contain different amounts of the impurities which yield a blue color. The label, or the cost, of a given brand is no criterion whatever as to the amount of color obtained from its fresh solutions. The best grades of sodium cyanide should nevertheless be used, because of their much smaller tendency to produce precipitates.

One qualification must be added to the statement made above to the effect that the cyanide solutions improve with age. The ammoniacal decomposition of the cyanide sooner or later destroys its efficiency. Strongly ammoniacal solutions do not yield the maximum color and show a greater tendency to produce turbidity. The accumulation of ammonia can be retarded by covering the container with a beaker instead of stoppering with a cork. The ammoniacal solutions can be restored to substantially their original value by boiling off the ammonia and then diluting to the original volume.

The blue color obtained from the cyanide and the uric acid reagent alone is much increased by the application of heat, and is much greater if heat (water bath) is applied at once than if the reaction is allowed to proceed for 2 to 3 minutes at room temperatures before heating. The reducing impurities in the cyanide solutions diminish spontaneously, at first rapidly, then more and more slowly, and many weeks must elapse before they are entirely gone. In 2 to 3 weeks the impurities have diminished to such an extent that practically no color is obtained in the cold, and the color produced on heating, after standing for 2 minutes at room temperatures, is so small as not to interfere with the uric acid determination.

The test for the blank due to impurities in the cyanide, is made as follows: Transfer 5 cc. of water, 2 drops of lithium sulfate solution, and 2 cc. of the 15 per cent cyanide solution to a test-tube. Add 1 cc. of the uric acid reagent, let stand for 2 minutes. The solution should remain colorless. Heat in boiling water for 1.5 minutes and cool; some color is obtained. To determine whether this color does or does not materially affect the uric acid values obtained in actual determinations, repeat the test described—with two graduated test-tubes and with standard uric acid solution—5 cc. in one, and 3 cc. plus 2 cc. of water, in the other. Dilute to volume after heating and compare the colors. If the cyanide is perfectly good, the weaker solution will give the theoretical reading, 33.5 mm., when the stronger is set at 20 mm.

The Uric Acid Determination.—Half fill a wide liter beaker with water and heat to boiling. Transfer 5 cc. of the blood filtrate and 2 cc. of water to one test-tube graduated at the 25 cc. mark, and transfer 5 cc. of the standard uric acid solution and 2 cc. of

water to another similar test-tube. Add 2 or 3 drops of 20 per cent lithium sulfate solution to each. From a burette add 2 cc. of the 15 per cent sodium cyanide solution. By means of a 5 cc. blood pipette,[9] or other graduated pipette, add 1 cc. of the uric acid reagent of Folin and Denis to each test-tube. Mix, and let stand for 2 minutes. At the end of 2 minutes transfer both test-tubes to the boiling water and leave them there for 80 seconds. Cool, dilute to volume, and make the color comparison in the usual manner, not omitting first to read the standard against itself. When the standard is set at 20 mm. 20 divided by the reading of the unknown, times 4, gives the uric acid content in milligrams per 100 cc. of blood. The proportionality of the color obtained is in this case so good, if the cyanide is right, that readings between 10 and 40 mm., covering a range of from 2 to 8 mg., are dependable.

It will be noted that the heating period is given in terms of seconds. The reason for this limitation is the greater tendency to get precipitates from prolonged heating, a tendency which is further increased by the evaporation within the tube, whereby a faint ring of precipitate is necessarily formed at the surface of the liquid. Heating for 60 seconds in boiling water is sufficient to give the maximum color. There is, therefore, no object in heating longer than the time prescribed.

It is obvious that the lithium sulfate and the sodium cyanide might be combined into one solution. I have refrained from prescribing such a combination because, for a time at least, one may have to deal with bloods containing added potassium oxalate as well as those containing lithium oxalate. If much potassium oxalate is present it may be necessary to use 4 drops of lithium sulfate to prevent precipitate formation.

The uric acid values obtained by the direct determination described above are often from 0.1 to 0.2 mg. higher than those obtained by the help of the silver lactate precipitation. In the course of my control determinations by means of silver lactate I have found that the original conditions described by Folin and Wu are not entirely dependable. Some of the uric acid present

[9] 5 cc. blood pipettes are very much better than ordinary pipettes, graduated in tenths of a cc., for many purposes. 5 and 10 cc. blood pipettes, made according to my specifications, can be obtained from the Emil Greiner Company, 55 Fulton Street, New York.

occasionally fails to go into the silver precipitate. It is, of course, essential that no such losses shall occur, especially when the precipitation is to be used as a standard control process for checking the results of the direct determination. I have therefore revised the precipitation process.

The silver lactate solution should be prepared as follows: Dissolve 100 gm. of silver lactate in about 700 cc. of warm water. To 100 cc. of 85 per cent lactic acid add 100 cc. of 10 per cent sodium hydroxide. Pour this partly neutralized lactic acid into the silver lactate solution, dilute to 1 liter, and set aside to allow the sediment, always present, to settle. Use only the clear supernatant solution. By thus neutralizing a part of the lactic acid, one provides for any excess acidity present in the blood filtrate and also for traces of mineral acids which may be present in some samples of silver lactate or in the lactic acid.

For some reason, the nature of which I do not know, it is necessary to add a very large excess of silver lactate solution in order always to get complete precipitation of the uric acid. The 2 cc. prescribed by Folin and Wu are not adequate to secure the quantitative recovery of uric acid added to sheep blood filtrate.

It should be stated that the precipitation of uric acid from ordinary solutions containing a little sodium chloride is an entirely different proposition from that of precipitating the uric acid from blood filtrates. It is, in fact, very doubtful whether it is possible to get 100 per cent recovery from water solutions when the uric acid and the sodium chloride content correspond to the concentrations found in blood filtrates. From the artificial solutions I get the best recovery (90 to 95 per cent) by adding only 1 cc. of the silver lactate, while in the case of the blood filtrate 100 per cent recovery is obtained by adding 7 cc. of the silver reagent.

To isolate the uric acid from the blood filtrate, transfer from 2 to 5 cc. (usually 5 cc.) of the latter to a centrifuge tube. With a blood pipette, or a small cylinder, add 7 cc. of the silver lactate solution. No stirring is necessary or desirable. Let settle for 1 to 2 minutes, and then centrifuge. All the uric acid, down to the last trace, will now be in the precipitate.

Decant the supernatant solution as completely as possible, and add 1 cc. of a 10 per cent solution of sodium chloride in tenth

normal hydrochloric acid. Stir *thoroughly* with a fine glass rod; add 4 cc. of water and stir again. Centrifuge.

Pour the supernatant solution, which need not be perfectly clear, into a test-tube graduated at the 25 cc. mark. Transfer 5 cc. of the standard uric acid solution to another similar test-tube. To the contents of each tube add 2 drops of lithium sulfate, 2 cc. of sodium cyanide, and 1 cc. of the uric acid reagent. Shake a moment and let stand for 2 minutes. Then heat for 80 seconds, cool, dilute to volume, and make the color comparison and the calculation as in the direct method.

It will be noted that the preliminary dilution of the solution with 2 cc. of water is omitted in this second process. That extra dilution is prescribed in the direct determination only as an added precaution against the formation of turbidity during the heating process. Personally I seldom add that water because I can nearly always avoid precipitate formation without it. The addition of the extra water diminishes somewhat the depth of the color obtained, but this is of no consequence for the color produced under the given conditions is anyhow much deeper than the color obtained in any other process.

THE COLORIMETRIC ESTIMATION OF CYSTINE IN URINE.

By JOSEPH M. LOONEY.

(*From the Biochemical Laboratories of Harvard Medical School, Boston.*)

In this paper I shall describe an application of the method for the colorimetric estimation of cystine recently advanced from this laboratory[1] to the quantitative determination of cystine in urine. The following procedure was devised to make available a method for the study of a series of cases of cystinuria which will be published in another paper.

The process depends on the fact that cystine reacts with phosphotungstic acid in the presence of sodium sulfite to give a deep blue color. Cystine alone, without the addition of sodium sulfite, does not give any color with the reagent. Uric acid and the other urinary constituents which reduce phosphotungstic acid under certain conditions, which will be given in detail later, give the same color whether sodium sulfite is present or absent. The amount of cystine present in urine is estimated by determining the increase in color of the specimen after the addition of sodium sulfite.

In order to determine the effect of sodium sulfite on the color produced by uric acid the following experiment was performed:

1 mg. of uric acid was placed in each of two 100 cc. volumetric flasks, and 20 cc. of saturated sodium carbonate were added to both. 1 cc. of 20 per cent lithium sulfate was then added to each flask to inhibit the formation of a precipitate of the reagent. 10 cc. of 20 per cent sodium sulfite were added to the first flask and the solutions in both flasks were well shaken. 3 cc. of phosphotungstic acid were now added to each flask, which were then shaken and allowed to stand for 5 minutes, and were then diluted to volume, mixed, and read in the colorimeter.

[1] Folin, O., and Looney, J. M., *J. Biol. Chem.*, 1922, li, 427.

There was no appreciable difference in the amount of color produced. Various repetitions of this experiment, in which the undiluted solutions were allowed to stand for different periods after the addition of the reagent before being diluted and read, showed that the sulfite decreases the speed of reoxidations so that the fading in the flask containing the sulfite is considerably diminished.

This effect does not appear until after about 10 minutes standing so that ample time is given to read the solutions. If allowed to stand less than 5 minutes the color in the flask which is free from sulfite is less than that in the other flask.

TABLE I.

Standard 2 cc. of urine + 20 cc. of sodium carbonate + 10 cc. of sodium sulfite set at 20.0 and read against 2 cc. of urine + 20 per cent sodium carbonate.

Time elapsed.	Readings.	Amount.	Loss.
min.			per cent
0	21.2	0.94	6.0
2	20.8	0.96	4.0
6	20.7	0.96	4.0
8	20.5	0.98	2.0
15	21.3	0.94	6.0
20	21.7	0.92	8.0
25	22.9	0.87	13.0
45	26.8	0.75	25.0
60	28.7	0.70	30.0

The same effect is given by normal urine as can be shown by the following experiment:

2 cc. of normal urine were placed in each of two 100 cc. volumetric flasks. To the first of these were added 20 cc. of saturated sodium carbonate and 10 cc. of 20 per cent sodium sulfite; to the second were added only 20 cc. of saturated sodium carbonate. Both flasks were then shaken and 3 cc. of phosphotungstic acid added, after which the flasks were again shaken, diluted to the mark, and well mixed.

The solution containing the sulfite was used as the standard and the second solution was read against it immediately and at intervals for 1 hour as shown in Table I.

The maximum color obtained from the urine not containing the sulfite was only 2 per cent less than that given by the one which contained 10 cc. of 20 per cent sodium sulfite and this difference may be assumed to be due to cystine present in normal urine.   The amount of cystine found in normal urine varies from nothing to about 10 mg. per 100 cc., averaging about 4 mg. per 100 cc.

The fact that the addition of sulfite does not alter the depth of color produced by uric acid contained in urine allows one to use a solution of cystine as the standard, and to determine the cystine content by subtracting the cystine equivalent of the urine before the addition of sulfite from that obtained after such addition.

The procedure which I have found most satisfactory follows:

The standard consists of a solution of pure cystine in 5 per cent sulfuric acid made up to contain 2 mg. of cystine in each cc. of solution.   This standard keeps indefinitely.   1 cc. of standard solution is taken for each determination.

1 cc. of standard solution containing 2 mg. of cystine is placed in a 100 cc. volumetric flask, 20 cc. of saturated sodium carbonate, 10 cc. of 20 per cent sodium sulfite, and 1 cc. of 20 per cent lithium sulfate are added and the solution is well mixed.

In a second flask from 1 to 10 cc. (usually 2 cc.) of urine are placed and treated in a similar manner.

In a third flask the same amount of urine is placed and treated as above except that no sulfite is added.

3 cc. of the uric acid reagent of Folin and Denis are now added to each flask and the flasks well shaken.   After standing 5 minutes the solutions are diluted to volume and mixed.   The solutions are read against the standard set at 20.0.   The time of reading must not be later than 8 minutes after the reagent is added and for this reason a new standard must be prepared for each determination.

The amount of cystine is found by subtracting the amount of reducing substances in the third flasks, calculated in mg. of cystine from the total color-producing substances after the addition of sulfite, found in the second flask, also calculated in mg. of cystine.

An example will make this clear.

Standard 2.0 mg. cystine set at 20.0.

Reading.

5 cc. of urine with   sodium sulfite   14.0 $\dfrac{20 \times 2}{14.0} = 2.86$ mg. cystine.

5 " "   " without   "   "   18.2 $\dfrac{20 \times 2}{18.2} = 2.20$ "   "

Cystine content of 5 cc. urine = 0.66 mg.

In certain cases in which there is much cystine it may be necessary to use a different amount of urine for the blank and total color readings so that the readings may come between the usual limits of 13.0 and 27.0. It is then necessary to reduce both amounts to the same quantity of urine before subtracting.

When the urine is so concentrated that cystine is precipitated it is necessary to separate the precipitate from the solution by the help of a centrifuge or by filtering. The precipitate is dissolved in a small amount of 5 per cent sulfuric acid and diluted to a definite volume. The two solutions are determined separately and the amounts combined to give total quantity.

That this method is accurate may be seen from the following experiment:

370 cc. of normal urine were placed in a strong flask and 500 mg. of cystine were added. The flask was placed in a shaking machine and agitated for 3 hours. The solution was then centrifuged and the precipitate dissolved in 5 per cent sulfuric acid and then diluted to 200 cc.

2 cc. of the clarified urine were then taken and the amount of cystine was estimated according to the procedure given above.

Standard 2 mg. cystine set at 20.0.

Reading.

2 cc. urine with   sulfite   14.5 $\dfrac{20.0}{14.5} = 1.38$ mg. per cc.

2 " "   without   "   17.3 $\dfrac{20.0}{17.3} = 1.16$ "   "   "

1 cc. urine contains 0.22 mg. cystine.

370 "   "   "   81.4 "   "

1 cc. of the solution, containing the precipitate, was then estimated in a corresponding manner.

Standard 2 mg. cystine set at 20 0.

Reading.

1 cc. of solution with   sulfite   19.0 $20.0 \times 2 = 2.10$ mg.
1 "   "   "   without   "   No color.   0.00

1 cc. of solution contains 2.10 mg. cystine.
200 "   "   "   "   420 0   "   "
Urine filtrate   "   81.4   "   "

                                   501.4   "   "
Cystine taken   500.0   "   "

If the urine contains albumin it must be removed before attempting to estimate the cystine. For this purpose I find that trichloroacetic acid gives good results and does not interfere with the subsequent estimation of cystine.

The procedure adopted to procure a protein-free filtrate is as follows: Accurately measure 50 cc. of urine into a 100 cc. volumetric flask, add 5 cc. of 20 per cent trichloroacetic acid, fill to mark with distilled water, stopper, and shake vigorously. Filter through a dry filter paper into a clean dry flask.

The figure obtained for this filtrate multiplied by 2 gives the amount for the untreated urine.

The results in Table II were obtained on normal urines to which known amounts of cystine had been added. The last three urines had also been treated by the addition of egg albumins.

TABLE II.

No.	Cystine added.	Cystine recovered.	Remarks.
	mg. per cc.	mg. per cc.	
1	0.50	0.51	
2	0.50	0.52	
3	1.00	1.03	
4	1.50	1.46	
5	1.50	1.48	
6	0.50	0.47	Albumin removed as described.
7	0.25	0.24	" " " "
8	1.00	0.98	" " " "

ON THE PROTEOLYTIC ENZYMES OF THE SPLEEN.

By S. G. HEDIN.

(*From the Laboratory of Physiological Chemistry, University of Upsala, Upsala, Sweden.*)

INTRODUCTION.

Since Salkowski in 1890 called attention to the fact that animal organs, when kept at 37° with antiseptics, undergo a change more or less similar to the digestion of proteins in the alimentary canal,[1] several investigators have been working along the same lines. Schwiening[2] found that Na_2CO_3 checks the process and Bondi[3] observed a favorable influence of HCl upon it in the case of the liver, which Rowland and I found to be the case with all the organs tried by us except the pancreas.[4] The process is generally called autolysis. Working with the spleen I found that this organ contains two proteolytic enzymes; one of them acting principally in an acid medium (β-protease) and one acting mainly in an alkaline medium (α-protease).[5] The latter could not be properly shown unless the spleen had been previously digested with a weak acid. The products obtained by the action in an acid medium were studied by Leathes,[6] who was able to isolate several amino-acids, and Cathcart[7] who was able to isolate amino-acids from the products resulting from the action of the α-protease. More recently the proteolytic enzymes of the spleen have been studied by Morse,[8] who confirmed the results of Hedin

---

[1] Salkowski, E., *Z. klin. Med.*, 1890, xvii, suppl., 77.

[2] Schwiening, H., *Virchows Arch. path. Anat.*, 1894, cxxxvi, 444.

[3] Bondi, C., *Virchows Arch. path. Anat.*, 1896, cxliv, 373.

[4] Hedin, S. G., and Rowland, S., *Z. physiol. Chem.*, 1901, xxxii, 341, 531.

[5] Hedin, S. G., *J. Physiol.*, 1904, xxx, 155.

[6] Leathes, J. B., *J. Physiol.*, 1902, xxvii, 360.

[7] Cathcart, E. P., *J. Physiol.*, 1904–05, xxxii, 299.

[8] Morse, M., *J. Biol. Chem.*, 1917, xxxi, 303.

but pointed out that the α-protease does not affect native proteins. Dernby,[9] who likewise confirms the existence of two enzymes, finds in all tissues "proteolytic enzymes of the type of trypsin or erepsin that attack only peptones or peptides" and have their optimal action at pH 7.8; in addition he assumes the "existence of pepsin-like enzymes, which split native proteins to peptones but not further" and have their optimal action at pH 3.5.

EXPERIMENTAL.

In all my experiments it has been very useful at first to act upon the spleen substance with an acid, either added to the spleen mass or formed during digestion. As will be seen later on a larger quantity of the enzymes is obtained through the action of a proper amount of acid, and in addition a very strong proteolytic activity sets in when the acid is present and the undissolved substance can easily be filtered off and washed. Without the previous action of the acid it is indeed very difficult to bring about any filtration of the spleen mass at all. I have carried out the treatment with acid and the subsequent extraction of the enzymes in the following way.

100 gm. of minced ox spleen are mixed with 300 gm. of water and 2.25 cc. of 20 per cent acetic acid. Chloroform and toluene are added to prevent bacterial action. After about 24 hours at 37° the contents of the bottle are filtered; Filtrate A is kept for further investigation, and the residue is thoroughly washed with water. Then the residue is mixed with 150 cc. of a casein solution, obtained by dissolving 20 gm. of casein (Hammarsten) in 400 cc. of water + 100 cc. of 0.1 N NaOH. Chloroform and toluene are again added and the whole is digested for 24 hours at 37° and then filtered. Filtrate B is investigated in the manner described below. The residue is thoroughly washed and then extracted with 5 per cent NaCl solution at 37° for 24 hours. Then the solution is filtered off and added with 30 gm. of Am₂SO₄ to 100 cc. of solution. The precipitate formed on the addition of the salt is filtered off and dialyzed after the addition of water. When the Am₂SO₄ has been nearly completely removed, the fluid is filtered from the undissolved proteins, the residue on the filter is dissolved in as little NaOH as possible and forms after filtration Solution C (to be described later on).

The above technique makes it possible to a certain extent to separate the different enzymes contained in the spleen mass. In

⁹ Dernby, K. G., *J. Biol. Chem.*, 1918, xxxv, 179.

addition to the two enzymes, α- and β-protease which I found
before, I have been able to demonstrate in the spleen the presence
of erepsin. The method of investigation just described is shown
in the following scheme.

Minced spleen is digested with acetic acid.

Filtrate A contains all enzymes.

Residue is extracted with casein solution.

Casein extract B mainly contains β-protease and erepsin.

Residue is extracted with NaCl solution. Solution C obtained as indicated above contains mainly α-protease.

Enzyme Action of Casein Extract B.

The action of the extract upon casein was carried out with
specimens made up in the following way:

20 cc. of casein extract + 25 cc. of casein solution + varying amounts
of 0.1 N NaOH or of 0.2 N HCl. Water was added to such an amount
that the volumes of the specimens belonging to the same series were the
same. The digestion lasted 3 days at 37°; then it was interrupted by
adding 35 cc. of tannic acid solution.[10] After 1 night the specimens were
filtered, 50 cc. of filtrate were taken for N determination and the result,
expressed in cc. of 0.1 N acid, after deduction of the figure obtained from
the control specimen with boiled casein extract, was calculated for the
whole volume of the specimens. The figures obtained in this way are
recorded in Table I. The casein solution, before being used for the extrac-
tion, shows an approximately neutral reaction, pH being 6.94; the casein
extract is decidedly more acid, pH being 6.5. The pH in this as well as
in all the following experiments was determined by the electrometric
method (18°).

The figures of the controls, corresponding to the digestion dur-
ing the extraction with casein, were: Experiment 1 = 34.45,
Experiment 2 = 24.95, Experiment 3 = 32.15, Experiment 4 =
30.95, and Experiment 5 = 32.15.

[10] The casein solution was made up as described on page 178 and the
tannic acid solution contained 100 gm. of tannic acid, 50 gm. of sodium
acetate, 50 gm. of NaCl, and 50 cc. of glacial acetic acid in 1 liter.

As can be seen, the strongest digestion took place in the specimens with 2 cc. of HCl.   In two experiments the concentration of the H ions was determined in the specimens with the strongest action and pH was found 5.7 for Experiment 4 and 5.45 for Experiment 5.   In the specimen without NaOH and HCl, pH equalled 6.7 and in the one with 2 cc. of NaOH, it equalled 7.3. In the specimen with 15 cc. of HCl, pH corresponds to the value 1.96.   Consequently the casein extract, when acting upon casein, shows its maximum effect in a weakly acid medium.   The action •

TABLE I.

Experiment.	1	2	3	4	5
cc.					
NaOH 4					1.85
3					2.55
2	6.05	7.3	10.0		2.65
1	10.00	10.8	14.15		2.90
0	16.85	14.3	18.45	15.85	5.20
HCl 1	30.15	19.1	27.25	25.4	10.2
2	34.45	20.95	30.6	28.6	13.6
3	32.65	20.8	27.25	25.1	13.6
4	20.70	18.7	21.35	20.45	11.65
5	19.15			19.35	
6				19.1	
7				18.25	
8				16.4	
9				14.4	
10	11.0			11.95	
15	0.3				

in an alkaline medium is much weaker and in a medium most favorable for the action of pepsin (pH 2) it has no effect at all. The determination of pH was always carried out before the digestion; the change of pH taking place in the course of the digestion has turned out to be very small, the casein present acting as a buffer.   In some experiments pH was determined before and after the digestion.   The results are recorded in Table II at the same time giving the amount of digestion determined as above.

There can hardly be any doubt that the action of the casein extract upon casein which has its maximum effect in a weakly

acid medium, depends mainly upon the presence of the β-protease
described by the author. But it is quite possible that other
enzymes are present as well. Since there is always some action
in a weakly alkaline medium some α-protease may be present.
In order to test whether there is any erepsin I tried the action of
the casein extract upon Witte's peptone. I removed from the
peptone the bulk of the products not precipitable by tannic acid,
in the following way: 20 gm. of Witte's peptone were dissolved
in 500 cc. of water and the solution after neutralization with HCl
was dialyzed for 3 days against tap water. Then the solution
was heated on the water bath for $\frac{1}{2}$ hour, filled up to 650 cc.,
and filtered. The reaction is now weakly alkaline, pH being
about 7.5. The experiments with peptone were carried out in

TABLE II.

Before digestion.	After digestion.	Amount of d gestion
7.55	7.55	7.1
7.55	7.58	5.1
7.10	7.12	8.2
6.60	6.57	13.35
6.61	6.54	11.45
6.08	6.06	23.0
6.08	6.01	14.9

the same way as those with casein except that 25 cc. of peptone
solution were added instead of 25 cc. of casein solution. In order
to study the effect of the same casein extract upon casein and
upon peptone the following experiment was carried out. The
specimens contained 20 cc. of casein extract + 25 cc. of casein
or peptone solution + 5 cc. made up of the recorded amount of
0.1 N NaOH or 0.2 N HCl and of water. The digestion lasted
3 days at 37°. To the casein specimens 35 cc. and to the peptone
specimens 30 cc. of tannic acid were added. Of the former
specimens 50 cc. of filtrate and of the latter 65 cc. were taken
for a N determination. The control specimen gave with casein
the value 32.1 and with peptone 53.55. After deduction of these
values the results were as given in Table III.

From these figures it can be seen that the strongest action
took place upon casein in an acid medium (pH 5.45) and upon

peptone in an alkaline one (pH 7.5).   The action upon casein in
an alkaline medium was very weak and in all probability it was
due to a small amount of α-protease being present.   From this
experiment it must, I believe, be inferred that the casein extract
contains in addition to the β-protease acting most strongly upon
casein in an acid medium, an enzyme hardly acting upon casein
at all but easily splitting up peptone; this action is most marked
in an alkaline medium.   In all probability this enzyme must be
termed an erepsin, although it differs from the erepsin found by
Cohnheim in the mucous membrane of the intestine inasmuch as
this enzyme acts upon casein as well as upon peptone.   Further

TABLE III.

	Casein.	Peptone.
cc.		
NaOH 4	1.85	
3	2.55	20.00
2	2.65	19.90 (pH 7.5)
1	2.90	19.60
0	5.20	18.80
HCl 1	10.20	16.65
2	13.60 (pH 5.45)	13.35
3	13.60	11.90
4	11.65	10.70
5		10.15

evidence of the existence of the erepsin in the spleen substance
will be given later on in this paper.

Vernon first called attention to the existence of ereptic enzymes
in animal tissues other than the intestine and pancreas[11] and
lately Dernby[9] has showed that several organs split up peptone,
but he did not decide whether this action was due to an erepsin
or to a tryptic enzyme.

Enzyme Action of Solution C.

The experiments on the action upon casein were carried out
with 10 cc. of Solution C + 25 cc. of casein solution in addition
to 4 cc. made up of the recorded volumes of 0.1 N NaOH or 0.2 N

[11] Vernon, H. M., *J. Physiol.*, 1904-05, xxxii, 33.

HCl and of water.   The results of two series of experiments are
recorded in Table IV.

The values of pH obtained from the corresponding specimens
of the two series are not quite the same, because the amount of
NaOH used for the preparation of Solution C was not and could
not be exactly the same in the two cases.   It is quite evident
from these two series that Solution C has its strongest action
upon casein in a medium decidedly alkaline, pH being 8.8 to
8.9.   Solution C, therefore mainly contains the α-protease pre-
viously found by me.   From the way in which Solution C
was obtained it can be seen that the α-protease adheres to pro-
teins, that, like globulins, are soluble in dilute NaCl solutions,

TABLE IV.

Series.	I	II
cc.		
NaOH 4	16.1 (pH 8.88)	
3	15.95 (pH 8.57)	7.4 (pH 8.79)
2	14.9	7.35 (pH 8.29)
1	13.7	7.0
0	11.95	6.25 (pH 7.35)
HCl 1	10.35	5.6 (pH 6.50)
2	6.5 (pH 5.73)	4.2 (pH 5.90)
3		3.35

not soluble in water, but soluble in alkali.   I have, therefore,
tried to extract the enzyme from the residue obtained after
the treatment of the spleen mass with acetic acid, by the
aid of MgO suspended in water.   After 24 hours at 37° the
mass was filtered and the pH of the filtrate was found to be
9.23.   This solution showed its maximum action upon casein at
about pH 5.5.   Therefore, the alkaline solution obtained with
MgO was precipitated with acetic acid, the precipitate was filtered
off and dissolved in as little NaOH as possible.   Then with this
solution and casein in the way described above the results shown
in Table V were obtained.

The maximum effect was therefore obtained at the same pH
as above with Solution C and the α-protease was evidently
present in the solution tried.

In my work on the spleen enzymes already referred to (page 177) I pointed out that the α-protease is checked in its action by the serum albumin of the serum, the β-protease not being checked. With the enzyme solutions obtained as above I have carried out experiments on this question. Specimens were prepared containing 10 cc. of Solution C + 5 cc. of serum albumin (ox) +25 cc. of casein solution +3 cc. of 0.1 N NaOH. The control contained 5 cc. of serum albumin boiled on the water bath for 30 minutes. The experiments were carried out as above. In one experiment,

TABLE V.

cc.	
NaOH 5	2.45
4	4.40
3	6.25 (pH 8.80)
2	4.95
1	4.15
0	3.85
HCl 1	4.65
2	4.25
3	3.75

where the pH of the above specimen was 8.57, the figures obtained were:

Serum albumin, not heated.................................. 3.7
    "   "   heated.....................................11.0

The inhibiting action of the serum albumin used, therefore, corresponds to 7.3. In another experiment the pH was 9.10 and the figures were:

Serum albumin, not heated................................3.45
    "   "   heated.....................................9.45

The inhibiting action, therefore, corresponded to 6.0. In another experiment with Solution C, HCl was added instead of NaOH and to such an amount that the pH of the specimen was 5.7:

Serum albumin, not heated..................................2.1
    "   "   heated.....................................3.7

The inhibiting action was, therefore, 1.6. In these experiments on the inhibiting action of the serum albumin the enzyme checked was α-protease, this enzyme being checked in alkaline and in acid media. As I have found before, the inhibiting substance of the serum is destroyed by the action of acid; but for such a destruction rather a strong action of the acid is required, and when the inhibiting substance has already been acting upon the enzyme, no enzyme can be liberated by a subsequent action of an acid.[12]

Solution C exercises some action upon Witte's peptone as well as upon casein, this action being checked by serum albumin. In one experiment the following figures were obtained. The specimens were made up as in the experiments with casein, peptone being substituted for casein.

	Experiment with	
	Peptone.	Casein.
Serum albumin, not heated....................	3.08	3.6
" " heated........................	5.85	6.4

In all my experiments on the influence of serum albumin upon the enzyme action of Solution C, I observed an inhibiting influence of the serum albumin. When I tried the influence of the serum albumin upon the action of the β-protease or the erepsin, I found either no influence or a weak one. In the cases of a weak inhibiting action I thought this was due to the enzyme solution tried, containing some α-protease.

Although the α-protease in all probability possesses the power of splitting peptone, it is able to carry out the beginning of the splitting process of casein to a greater extent than the end of the same process; this is borne out by the fact that the α-protease and the erepsin, when acting together upon casein, digest the same to a greater extent than they both do when acting separately. This can be seen from the following experiment, where the α-protease is represented by Solution C and the erepsin by Filtrate A (strongly dialyzed) which sometimes contains rather a large amount of this enzyme and only a little α-protease.

[12] Hedin, S. G., Festskrift Olof Hammarsten, *Upsala Läkaref. Förh.*, suppl., 1906, xi.

No		pH
1	10 cc. of Solution C + 10 cc. of Filtrate A + 25 cc. of casein + 4 cc. of 0.1 N NaOH..	9.07
2	10 cc. of Solution C + 10 cc. of Filtrate A heated + 25 cc. of casein + 4 cc. of 0.1 N NaOH...........................	9 07
3	10 cc. of Solution C heated + 10 cc. of Filtrate A + 25 cc. of casein + 4 cc. of 0.1 N NaOH.............	9.07
4	Control with Solution C and Filtrate A heated................	9.07

3 days at 37°; 35 cc. of tannic acid solution; 50 cc. of filtrate

No.	Figures obtained.	Control (4 4) deducted.
1	18.5	17.4
2	9.4	8.3
3	2.0	0.9
4	1.1	

The figures obtained from the separate action of both enzymes being 8.3 and 0.9, the sum of these (9.2) is far exceeded by the figure 17.4 representing the common action of both enzymes. This experiment shows at the same time that the α-protease alone exercised rather a strong action upon casein and the erepsin alone hardly any at all. Another experiment showed that the erepsin solution readily acted upon Witte's peptone:

No.	
1	10 cc. of Filtrate A + 25 cc. of peptone + 2 cc. of 0 1 N NaOH
2	10 " " " A heated + 25 cc. of peptone + 2 cc. of 0.1 N NaOH

After digestion as above the following figures were obtained:

No.	Figures obtained.	Control (9 95) deducted.
1	19.10	Effect = 9.15
2	9.95	

In another experiment with α-protease and erepsin, casein extract B was used as erepsin solution. The figures were, after deduction of the control value:

α-Protease + erepsin........ ....14.4
"   alone.. 7.5
Erepsin alone... 1.6

Further Experiments on Filtrate A.

In addition to crepsin, Filtrate A contains mainly β-protease and varying amounts of α-protease. In the following experiments, Filtrate A was half saturated with Am_2SO_4 by adding 30 gm. of this salt to 100 cc. of fluid. The precipitate formed was filtered off and dialyzed with as little water as possible till the salt was removed. The solution thus obtained was used for determining the action upon casein at different values of pH. Each specimen contained 20 cc. of enzyme solution + 25 cc. of casein solution +5 cc. made up of water and 0.1 N NaOH or 0.2 N HCl. The figures obtained were:

		Experiment 1.	Experiment 2.	
	cc.			
NaOH	5	4.35 (pH 9.79)	1.8	(pH 9.72)
	4	5.6	4.25	
	3	7.22	12.3	
	2		19.4	(pH 8.19)
	1	8.41	24.2	(pH 7.56)
	0	8.5	29.15	(pH 7.25)
HCl	1	11.20 (pH 6.43)	38.25	(pH 6.36)
	2	9.85	33.3	(pH 5.85)
	3	8.85	29.6	
	4	6.65	23.95	
	5	4.40		

In these series the strongest action is obtained in a medium slightly less acid (pH 6.4) than in the experiments with the casein extract recorded above. This may be due to the solution containing more α-protease than the casein extract, as shown by rather a strong digestion taking place in an alkaline medium, particularly in Experiment 2.

I have tested the influence of acid upon the enzymes present in Filtrate A in the following way. The filtrate derived from 1,000 gm. of ox spleen was precipitated by adding 500 gm. of Am_2SO_4 to 1 liter of filtrate, whereupon the precipitate was filtered off and dialyzed free from salt. The solution was then diluted with water to 1,000 cc. The enzyme solution thus obtained was treated with acid in such a way that 100 cc. of solution were kept with 15 cc. of 0.2 N HCl at room temperature for

24 hours (pH 2.04). This solution gave no action upon casein either in an alkaline or in an acid medium nor did it act upon peptone in an alkaline medium and, therefore, the three enzymes had been completely destroyed by the action of the acid.

The treatment with acid was, therefore, repeated with less acid: 100 cc. of solution + 10 cc. of 0.2 N HCl (pH 3.25) were kept at room temperature for 24 hours and then 20 cc. of 0.1 N NaOH were added (A). In the control specimen 100 cc. of solution were mixed with 10 cc. of acid and 20 cc. of NaOH and kept for 24 hours at room temperature (B). The specimens for digestion were prepared as follows:

No.		pH
1	(10 A + 10 B) heated + 25 casein solution + 3 NaOH	8.2
2	10 A (heated) + 10 B + 25 " " + 3 "	
3	(10 A + 10 B) heated + 25 " " + 3 "	
4	(10 A + 10 B) " + 25 peptone solution + 3 NaOH	8.3
5	10 A (heated) + 10 B + 25 " " + 3 "	
6	(10 A + 10 B) heated + 25 " " + 3 "	
7	(10 A + 10 B) " + 25 casein solution + 2 HCl	5.8
8	10 A (heated) + 10 B + 25 " " + 2 "	
9	(10 A + 10 B) heated + 25 " " + 2 "	

3 days at 37°; 30 cc. tannic acid : 50 cc. filtrate.

The figures obtained were:

No		After deduction of controls.
1	3.35	1.2
2	9.2	7.05
3	2.15	
4	10.9	1.5
5	24.7	15.3
6	9.4	
7	11.60	9.5
8	20.65	18.55
9	2.1	

In another experiment with the same enzyme solution the amount of acid used was 5 cc. and the amount of NaOH, 10 cc. The digestion was carried out in the same way as above and the figures obtained were:

No.		After deduction of controls.
1	7.85	5.35
2	9.95	7.45
3	2.5	
4	22.4	12.85
5	25.2	15.65
6	9.55	
7	20.5	18.05
8	21.0	18.55
9	2.45	

As can be seen from these experiments, the α-protease and the erepsin were weakened by the action of 5 cc. of 0.2 N HCl to 100 cc. of solution while the β-protease was hardly affected at all. By the action of 10 cc. of acid (pH 3.25) the two former enzymes were almost completely destroyed and the latter was evidently somewhat weakened.

The experiment with 10 cc. of acid to 100 cc. of enzyme solution was therefore repeated and the action of the resulting solutions, A and B, upon casein and peptone was tested at a different pH. Each specimen contained 10 cc. of enzyme solution (A or B) + 25 cc. of casein (or peptone) solution + 5 cc. made up of the recorded amount of 0.1 N NaOH or 0.2 N HCl and water. The digestion lasted 3 days at 37°, 30 cc. of tannic acid were added and 50 cc. of filtrate were taken for N determination. The controls with casein gave for A and B the figures 3.1 and 0.95 and those with peptone, 11.25 and 8.75. After deduction of these values the results were:

		Casein.		Peptone.	
		A	B	A	B
cc.					
NaOH	4	1.65	5.4	2.65	18.95
	3	2.5	9.35	3.55	19.7
	2	3.15	12.85	4.5	19.85 (pH 8.46)
	1	3.6	15.5	5.25	19.55
	0	4.95	19.85	6.7	19.6
HCl	1	10.7	26.0 (pH 6.32)	8.8	17.75
	2	14.1 (pH 5.24)	25.5	10.85	
	3	13.9	21.15	11.25(pH 4.34)	16.25
	4	12.5	19.85	10.75	14.75
	5	11.75	17.15	9.55	13 45

These figures show that without preceding treatment with acid (B) the strongest action took place upon casein in a weakly acid medium (pH 6.32) and upon peptone in a decidedly alkaline medium (pH 8.46). By the treatment with acid (A) the reaction most favorable for the action upon casein was somewhat moved to the acid side (pH 5.24) and the pH most favorable for the action upon peptone was moved from the alkaline side to the acid one (pH 4.34). The treatment with acid in all probability affects all the three enzymes but the effect upon the erepsin in this case is the most striking. In addition we find from the figures obtained that after the treatment with acid the reaction most favorable for the action upon casein and upon peptone is nearly the same (pH 5.24 and 4.34). The enzyme acting mainly at an acid reaction seems therefore to act upon peptone as well as upon casein. Consequently I find no reason for thinking that the digestion of proteins in an acid medium by the influence of spleen enzymes is caused by any kind of pepsin, this latter view in addition not being consistent with the fact that the enzymes are completely destroyed in a medium most suited to the action of pepsin (pH 2).

Effect of Acid upon the Spleen Substance.

As I have already pointed out, the autolysis of organs goes much faster in an acid medium than it does in an alkaline one. Some 15 years ago I found that the autolysis of the spleen and of other organs in an alkaline medium takes place much faster, if the organs are treated with a weak acid before the reaction is made alkaline, than it does if no treatment with acid takes place.[12]

I interpreted this fact as being due to the acid destroying a substance, which, if active, inhibits the action of the α-protease of the organs. This view was supported by the fact that the digestion of casein by means of spleen enzymes in an alkaline medium was checked by the serum albumin fraction of the serum and that this checking influence disappeared when the serum albumin fraction was treated with a weak acid.

Dernby[9] tries to explain the effect of pretreatment with acid upon the subsequent autolysis in an alkaline medium by assuming that in the course of the treatment with acid the substance of the organs is split up mainly into albumin and peptones and not

further, these products forming the proper substrate for the enzymes acting in an alkaline medium. This certainly cannot be the only reason for the autolysis in an alkaline medium being stronger after pretreatment with acid. This I believe to be proved by the following experiments.

Experiment 1.—Three specimens of the following composition were prepared: 25 gm. of minced spleen + 50 cc. of H_2O + MgO sufficient to keep the reaction slightly alkaline; and three specimens were made up of 25 gm. of spleen + 50 cc. of H_2O + 3.6 cc. of 2.5 per cent acetic acid. The specimens with MgO are termed A and those with acid, B. The whole is kept at 37° for 1 day with chloroform and toluene. Then two specimens of each kind were heated upon the water bath for 30 minutes, after which three new specimens were made up as follows:

No.	
1	$(A + B)$ heated + 15 cc. of H_2O + MgO
2	A heated + B + 15 " " " + "
3	$(A + B)$ heated + 15 " " " + "

The 15 cc. of H_2O were used for washing down the substance from one bottle into the other. After 2 days at 37° 50 cc. of tannic acid were added and 75 cc. of filtrate were taken for N determination. The results were:

Specimen.		Control (110.4) deducted.
1	125.2	14.8
2	155.0	44.6
3	110.4	

It will easily be understood that at the beginning the digestion specimens, Nos. 1, 2, and 3 contained the same quantity of proteins, albumoses, and peptones, half the quantity of those in Specimens 1 and 2 having been heated to destroy the enzymes and the whole amount having been heated in Specimen 3. The substrate was therefore practically the same in Specimens 1 and 2, but in Specimen 1 the enzymes present in the spleen kept in a weakly alkaline medium were active and in Specimen 2 the enzymes present after treatment with acid were active. In special specimens prepared in the same way as Specimens 1 and 2 the pH was determined after filtration and was found to be 8.0

for Specimen 1 and 7.93 for Specimen 2.   As can be seen from the figures obtained, the enzyme action at an alkaline reaction was very much stronger in Specimen 2 than in Specimen 1.

Experiment 2.—In this experiment, carried out in the same way as the one just recorded, the final figures, after deduction of the control, were

No.	
1	5.6
2	44.3

Experiment 3.—In this experiment Specimens A contained $CaCO_3$ instead of MgO as in the preceding experiments.   Therefore, the reaction in these specimens was approximately neutral.   Specimens B were acid as before. After 24 hours at 37° Specimens 1, 2, and 3 were prepared as before; except $CaCO_3$ was added instead of MgO.   In addition other specimens (Specimens 4, 5, and 6) were prepared quite like Specimens 1, 2, and 3, except 50 cc. of casein solution were added to each.   After 2 days at 37° 20 cc. of tannic acid were added to Specimens 1, 2, and 3, and 50 cc. to Specimens 4, 5, and 6.   50 cc. of filtrate were taken for N determination from Specimens 1, 2, 3 and 100 cc. from Specimens 4, 5, and 6.   After deduction of the controls the results were:

No.	
1	2.3
2	8.9
4	14.7
5	38.5

The figures show that the digestion in a neutral medium caused by the specimens that were previously kept with acid is stronger than that caused by the specimens kept in a neutral medium. The same difference is found if casein is added as a substrate as is shown by the values obtained in Experiments 4 and 5.

Experiment 4.—In this experiment $CaCO_3$ was added to Specimens A and no acid was added to Specimens B.   After 1 day at 37°, therefore, the reaction in Specimen A was approximately neutral (pH 6.9) and in Specimen B weakly acid (pH 6.5).   Specimens 1, 2, and 3 were prepared as above

and the values obtained show that even the small amount of acid produced
by the organ itself was sufficient to secure a larger amount of enzyme in B
than in A.

No.	
1	20.8
2	34.3

All the experiments recorded go to show that after a preceding
treatment with acid the amount of enzyme present acting in an
alkaline or even a neutral medium is larger than without such a
previous treatment. And since the quantity of albumoses and
peptones was the same in both cases, this cannot be explained in
the way suggested by Dernby.

In a set of experiments I have tested whether the solution of
spleen enzymes obtained by acting upon the spleen substance
for 24 hours at 37° with weakly alkaline or neutral water shows
any increase of activity after treatment with acid, but all my
attempts in this direction have failed to give any results. In
most cases some enzyme acting in an alkaline medium seems to
be decomposed by the action of the acid, and this destruction
of enzyme takes place even if no acid is added and the acid reaction
is produced simply by dialyzing the enzyme solution against
distilled water; in this way pH can be brought down to about 6.0.

The presence of the solid spleen substance seems therefore to
be necessary for the demonstration of the effect of acid found in
Experiments 1 to 4. I have tried to find out whether any differ-
ence in the effect of the acid is produced if the spleen substance
is kept in water for some time before the acid is added. One of
these experiments was carried out in the following way:

Experiment 5.—Two sets of specimens were prepared, one (A) contain-
ing 100 gm. of minced spleen substance + 300 cc. of H_2O; and the other
(B) with 2.25 cc. of 20 per cent acetic acid in addition to the constituents
of A. Both were kept at 37° for 24 hours; then 2.25 cc. of acetic acid were
added to A and these specimens were kept at 37° for another 24 hours, while
B was directly filtered and a given part of the filtrate was dialyzed against
tap water. After 24 hours at 37° A was filtered and the same volume of
the filtrate as of B was dialyzed. It should be noted that the filtration of
B took place markedly faster than the filtration of A. After the dialysis
the filtrates (A and B) were filled up to the same volume (200 cc. before
dialysis = 253 cc. after dialysis). Before dialysis pH was found to be

5.09 for A and 5.14 for B and after dialysis was about 8 for both. The amount of non-precipitable N in Filtrates A and B was determined by mixing 20 cc. of undialyzed filtrate with 10 cc. of tannic acid and taking 20 cc. of filtrate for N determination. The result was:

    For Filtrate A...25.0
      "   "   B...25.5

The residues of A and B obtained on filtering were thoroughly washed and extracted with casein as described on page 178. The amounts of enzymes present in dialyzed Filtrates A and B as well as those present in casein extracts A and B were compared.

TABLE VI.

3 days digestion; 40 cc. of tannic acid; 75 cc. of filtrate.

	pH	Result.	After deduction of controls.
(20 A + 20 B) heated + 25 casein solution + 2 NaOH, 0.1 N...	8.2	7.95	4.45
20 A heated + 20 B + 25 casein solution + 2 NaOH, 0.1 N...		15.6	12.1
(20 A + 20 B) heated + 25 casein solution + 2 NaOH, 0.1 N...		3.5	
(20 A + 20 B) heated + 25 peptone solution + 2 NaOH, 0.1 N...	8.0	17.6	7.6
20 A heated + 20 B + 25 peptone solution + 2 NaOH, 0.1 N...		22.7	12.7
(20 A + 20 B) heated + 25 peptone solution + 2 NaOH, 0.1 N...		10.0	
(20 A + 20 B) heated + 25 casein solution + 2.5 HCl, 0.2 N...	5.36	8.9	5.4
20 A heated + 20 B + 25 casein solution + 2.5 HCl, 0.2 N.........		15.8	12.3
(20 A + 20 B) heated + 25 casein solution + 2.5 HCl, 0.2 N...		3.5	

The results of the experiments with the filtrates are given in Table VI.

All these figures show that all the specimens where Filtrate B was active (not heated) contained considerably greater amounts of enzyme than the corresponding specimens where Filtrate A was acting. Since the enzyme solution that was not active was

always present as heated the substrate in the two specimens to be compared was always the same. The values obtained with casein as substrate in an alkaline medium show that Filtrate B contained considerably more α-protease than Filtrate A; the specimens with peptone in an alkaline medium do not allow of any conclusions as to the amounts of erepsin, since the results can be explained as caused by the α-protease, but the results obtained with casein in an acid medium make it very likely that the β-protease is present in a larger amount in Filtrate B than in Filtrate A.

In the experiments with the casein extracts the specimens were made up of 20 cc. of casein extract A or B + 25 cc. of casein

TABLE VII.

cc.	Casein.		Peptone.	
	A	B	A	B
NaOH 3	1.35	1.5	8.2	12.55
2	1.2	1.55	8.35 (pH 7.56)	12.45 (pH 7.50)
1	1.35	1.7	7.95	12.25
0	2.35	2.75	7.55	11.75
HCl 1	4.3	6.0	6.1	10.4
2	4.95 (pH 5.45)	8.0 (pH 5.45)	5.05	8.35
3	4.75	8.0	4.6	7.45
4	4.5	6.85	4.75	6.7
5			4.15	6.35

solution or peptone solution and varying amounts of 0.1 N NaOH or 0.2 N HCl, a proper amount of water making the volume the same in all specimens. The results are to be seen from Table VII.

The control values that have been deducted from the original figures were for casein A 11.85, B 18.9; and for peptone A 23.35 and B 33.35. From the above figures obtained with casein as a substrate it is evident that the strongest digestion in A and B takes place at the same pH but that the effect of A is decidedly weaker than the effect of B. Therefore, the amount of β-protease is considerably less in A than in B. The digestion of casein in an alkaline medium being very weak, there can only be very little

α-protease present in the casein extracts. The action of the casein extracts upon peptone having its maximum in an alkaline medium indicates the presence of erepsin in the casein extracts. The strongest action takes place at the same pH in both cases but it is considerably weaker in A than in B.

All the analyses carried out in the course of this experiment go to show that on immediate addition of a proper amount of acetic acid to the spleen substance more enzymes are secured than if the addition of acid takes place after the lapse of 1 day. And this seems to be the case not only concerning the α-protease but

TABLE VIII.

	A	B	pH
cc.			
NaOH 2	1.7	5 55	7.3
1	2 8	7.85	
0	4 2	10.25	6.68
HCl 1	6 8	15 3	
2	8.75	17.0	5.7
3	8.65	15.15	
4	7.6	11.85	

concerning the β-protease and the erepsin as well, and this difference is to be found in the solution obtained by the action of the acid as well as in the casein extract of the residue. The amount of α-protease present in the solution termed Solution C was in this experiment a little higher in the solution derived from B than from A, but the difference was not so material as in the other solutions.

Experiment 6.—In another experiment on the same subject only the casein extracts A and B were tested as to their action upon casein. The specimens were made up of 20 cc. of casein extract A or B + 25 cc. of casein solution + the recorded amount of 0.1 N NaOH or 0.2 N HCl and water up to the same volume in all specimens 3 days at 37°; 35 cc. of tannic acid; 50 cc. of filtrate. The control values deducted were 10.7 for A and 17.85 for B (see Table VIII).

The strongest action took place with the same amount of acid (2 cc.) in A and in B corresponding to pH 5.7. In this experi-

ment some α-protease seems to have been present in the casein
extract, this enzyme as well as the β-protease being strongly
reduced in casein extract A. This experiment, therefore, gives
the same result as the preceding one.

Experiment 7.—In this experiment one set of specimens (A₁) was made up
as follows: 100 gm. of minced spleen + 300 cc. of H_2O + 10 cc. of 0.1 N
NaOH. After 24 hours at 37°, 5 cc. of 0.2 N HCl and 2.25 cc. of 20 per cent
acetic acid were added and A₁ was left at 37° for another 24 hours; it was
then filtered and in every respect treated as was A in Experiment 5. Two
other sets of specimens, A₂ and B, were treated exactly as were A and B in
Experiment 5. In B, therefore, the acetic acid was added directly and in
A₁ and A₂ after 24 hours at 37°. To A₁ 10 cc. of NaOH were added directly
in order partly to neutralize the acid formed in the tissue in the course of
the first 24 hours at 37°. This amount of NaOH was neutralized before
the addition of the acetic acid. Consequently the reaction in A₁ during
the first 24 hours was kept less acid than in A₂. pH was determined in the
filtrates after 24 hours at 37°, after 24 hours with acid, and after dialysis
of the acid filtrates.

	A_1	A_2	B
After 24 hours at 37°.............	6.82	6.5	
" 24 " with acid at 37°................	5.22	4.14	5.16
" dialysis..................................	7.76	8.04	8.14

The amount of N was determined in the filtrates after 24 hours
at 37° and after 24 hours with acid by mixing 20 cc. of filtrate
with 10 cc. of tannic acid and taking 15 cc. of filtrate for the
determination.

	A_1	A_2	B
After 24 hours at 37°.........................	11.4	13.6	
" 24 " with acid at 37°................	19	21.1	25.15

Specimens were prepared containing the dialyzed filtrates, A₁
and B, casein or peptone, and NaOH or HCl exactly as indicated
above in Experiment 5 and the test for enzymes was carried out
in the same way.

	Resulting values.	After deduction of controls
(20 A$_1$ + 20 B) heated + 25 casein solution + 2 NaOH	4.1	1.7
20 A$_1$ heated + 20 B + 25 " " + 2 "	15.8	13.4
(20 A$_1$ + 20 B) heated + 25 " " + 2 "	2.4	
(20 A$_1$ + 20 B) " + 25 peptone solution + 2 "	18.45	4.9
20 A$_1$ heated + 20 B + 25 " " + 2 "	25.35	11.8
(20 A$_1$ + 20 B) heated + 25 " " + 2 "	13.55	
(20 A$_1$ + 20 B) " + 25 casein solution + 2.25 HCl	7.3	4.9
20 A$_1$ heated + 20 B + 25 " " + 2.25 "	15.1	12.7
(20 A$_1$ + 20 B) heated + 25 " " + 2.25 "	2.4	
(20 A$_1$ + 20 B) " + 25 peptone solution + 2.25 "	14.7	1.15
20 A$_1$ heated + 20 B + 25 " " + 2.25 "	20.1	6.55
(20 A$_1$ + 20 B) heated + 25 " " + 2.25 "	13.55	

The corresponding analysis with A$_2$ and B gave the following results:

	Resulting values.	After deduction of controls.
(20 A$_2$ + 20 B) heated + 25 casein solution + 2 NaOH	6.3	3.95
20 A$_2$ heated + 20 B + 25 " " + 2 "	15.75	13.4
(20 A$_2$ + 20 B) heated + 25 " " + 2 "	2.35	
(20 A$_2$ + 20 B) " + 25 peptone solution + 2 "	17.8	4.3
20 A$_2$ heated + 20 B + 25 " " + 2 "	25.6	12.1
(20 A$_2$ + 20 B) heated + 25 " " + 2 "	13.5	
(20 A$_2$ + 20 B) " + 25 casein solution + 2.25 HCl	7.65	5.3
20 A$_2$ heated + 20 B + 25 " " + 2.25 "	15.1	12.75
(20 A$_2$ + 20 B) heated + 25 " " + 2.25 "	2.35	
(20 A$_2$ + 20 B) " + 25 peptone solution + 2.25 "	15.3	1.8
20 A$_2$ heated + 20 B + 25 " " + 2.25 "	19.4	5.9
(20 A$_2$ + 20 B) heated + 25 " " + 2.25 "	13.5	

pH was in the specimens with casein and 2 NaOH = 8.2
"   "   "   "   "   " peptone " 2   "   = 7.86
"   "   "   "   "   " casein and 2.25 HCl = 5.74

These values of pH were very nearly the same in the corresponding specimens of the two series. From the values in the last column it can be seen that the quantity of enzymes present in B was everywhere larger than that in A$_1$ and A$_2$. The latter as a rule have given roughly the same values, except for the

values corresponding to the action of the α-protease; this value for A_1 was 1.7 and for A_2, 3.95. This enzyme was therefore decidedly more reduced in A_1 than in A_2, in all probability because the reaction during the first 24 hours was less acid in A_1 than in A_2. In addition it should be noted that the digestion of casein by B was a little stronger in the alkaline medium (pH 8.2) than in the acid one (pH 5.74); while the digestion caused by A_1 and A_2 was more powerful in the acid medium.

The casein extracts in this experiment gave the following values for pH: A_1, 6.14; A_2, 6.06; B, 6.04.

The quantities of enzymes investigated were as follows:

	Resulting values.	After deduction of controls.
(20 A_1 + 20 B) heated + 25 casein solution + 6 NaOH	32.8	2.0
20 A_1 heated + 20 B + 25 " " + 6 "	33.2	2.4
(20 A_1 + 20 B) heated + 25 " " + 6 "	30.8	
20 A_1 + 20 B " + 25 peptone solution + 6 "	45.95	7.2
20 A_1 heated + 20 B + 25 " " + 6 "	49.75	11.0
(20 A_1 + 20 B) heated + 25 " " + 6 "	38.75	
(20 A_1 + 20 B) " + 25 casein solution + 2 HCl + 4 H_2O	33.75	2.95
20 A_1 heated + 20 B + 25 " " + 2 "	39.2	8.4
(20 A_1 + 20 B) heated + 25 " " + 2 "	30.8	

In the same way A_2 and B were combined:

(20 A_2 + 20 B) heated + 25 casein solution + 6 NaOH	32.8	1.0
20 A_2 heated + 20 B + 25 " " + 6 "	34.5	2.7
(20 A_2 + 20 B) heated + 25 " " + 6 "	31.8	
20 A_2 + 20 B heated + 25 peptone solution + 6 "	45.6	6.2
20 A_2 heated + 20 B + 25 " " + 6 "	51.5	12.1
(20 A_2 + 20 B) heated + 25 " " + 6 "	39.4	
(20 A_2 + 20 B) " + 25 casein solution + 2 HCl + 4 H_2O	35.25	3.45
20 A_2 heated + 20 B + 25 " " + 2 " + 4 "	40.7	8.9
(20 A_2 + 20 B) heated + 25 " " + 2 " + 4 "	31.8	

pH was in the specimens with casein solution and 6 NaOH = 7.84
" " " " " " peptone solution and 6 NaOH = 7.66
" " " " " " casein solution and 2 HCl = 5.33

The digestion lasted 3 days. 40 cc. of tannic acid were added and 75 cc. of filtrate were taken for N determination.

As usual the figures show that only a small amount of α-protease is present in the casein extracts. But the amounts present are less in A_1 and A_2 than in B. The figures representing the action of erepsin and of β-protease likewise show that those enzymes are present in greater quantities in B than in A_1 and A_2.

Experiments show that more enzymes are obtained from the spleen if a proper amount of acid is added directly to the minced spleen mass than if the same amount of acid is added when the spleen mass has been kept in water for 24 hours at 37° at an alkaline, neutral, or even weakly acid reaction. One might try to explain this phenomenon in two ways:

1. The enzymes might be present in the organ in an inactive state, which could be made active by the acid. This explanation is not consistent with the fact that only small amounts of enzymes become active when the spleen mass has been kept for some time.

2. The enzymes present in the spleen are in some way deprived of their activity when the spleen is kept, and this process is prevented by the action of the acid. As soon as the enzymes have been deprived of their active power, they cannot be made active again by the acid; and if the acid has been acting for a proper time upon the fresh spleen, the enzymes do not lose their active power in a neutral or even alkaline medium. There does not seem to me to be any objection to this explanation and all the observations recorded in this paper can be explained in this way.

As to the way in which the enzymes are deprived of their active power nothing can be stated for certain. The action of the α-protease is inhibited by the serum albumin fraction obtained from the blood, and it seems to be quite possible that the inhibiting substance in the serum may be present in the spleen as well. This might account for the α-protease losing its activity, but since the serum albumin fraction does not exercise any definite checking action upon the β-protease and upon the erepsin, the vanishing of these enzymes cannot be explained in this way. Other substances inhibiting the action of these enzymes might be present in the spleen, but since the existence of such substances has not been demonstrated, I cannot offer any definite view on this subject.

CONCLUSIONS.

The main results of my experiments on the proteolytic enzymes present in the spleen of the ox may be summarized as follows:

1. The spleen contains at least three different enzymes: (1) α-protease, acting upon the spleen substance and upon casein in an alkaline medium (pH 8.8); (2) β-protease acting upon the spleen substance and upon casein in a weakly acid medium (pH about 5.4); and (3) erepsin not acting upon casein but having its most powerful action upon Witte's peptone at pH 7.5 to 8.5.

2. When the fresh spleen substance is kept in an alkaline, neutral, or even very weakly acid medium (pH 6.5), the enzymes lose much of their power of action, but this process is prevented by the presence of a little acid (*e.g.* pH 5.2).

3. A watery extract of the spleen obtained at about pH 5.2 contains varying quantities of all the enzymes referred to.

4. A casein extract of the residue obtained on filtering off the acid solution, contains mainly β-protease and erepsin.

5. If the residue obtained on filtering off the casein extract is extracted with 5 per cent NaCl or MgO, the part of the extracted substances not soluble in water contains mainly α-protease.

The above experiments were carried out with the spleen of the ox. The spleen of the horse gives, on the whole, the same results as far as my experience goes. Working in the same way as described above I found the most effective action of the casein extract in the specimen containing 20 cc. of casein extract + 25 cc. of casein solution + 4 cc. of 0.2 N HCl corresponding to pH 4.93. This concentration of the H ions lies decidedly more to the acid side than was found with the spleen of the ox. It should be noted that the addition of the acid in this case produced rather a voluminous precipitate of casein and that the pH was determined after this precipitate had been removed.

Having experimented upon the spleen mass with 1.7 cc. of 20 per cent acetic acid to 100 gm. of spleen + 300 cc. of H_2O for 1 day at 37°, I did not succeed in demonstrating the presence of the α-protease in the solution termed Solution C above, but using 2.5 cc. of the acid to the same amounts of spleen and H_2O, I succeeded. The most effective action of the α-protease upon casein took place at pH 9.31 or more to the alkaline side than in the experiments with the spleen of the ox.

I have carried out some experiments with the spleen of the pig as well. The most important difference that I have found between the spleen of the pig and that of the ox is that the amount of acid used for the original treatment of the spleen mass should not exceed 1 cc. of 20 per cent acetic acid to 100 gm. of spleen and 300 cc. of H_2O. More acid seems to be somewhat injurious to the enzymes. An experiment exactly like Experiment 3 (page 192) gave the same result and, therefore, the presence of acid prevents the enzymes from losing their active power.

EXPERIMENTAL RICKETS IN RATS.

IX. THE DISTRIBUTION OF PHOSPHORUS AND CALCIUM BETWEEN THE SKELETON AND SOFT PARTS OF RATS ON RACHITIC AND NON-RACHITIC DIETS.

By GERTRUDE E. McCANN and MARION BARNETT.

(*From the Department of Pathology of the College of Physicians and Surgeons, Columbia University, New York.*)

In experimental studies of rickets it was found (1) that rats fed certain diets, which were poor in phosphorus but rich in calcium content, developed rickets, while increasing the phosphorus in these diets above certain levels invariably prevented this disease. Treatment with cod liver oil (2) or with light (3) also prevented the development of rickets in rats fed these diets without the addition of phosphorus.

It seemed of interest to determine what occurred when diets were given which were extremely poor in phosphorus or in calcium; whether the bones and soft tissues shared the amount proportionally, or whether a point could be reached at which one or the other would suffer the greater deprivation. It was thought that some knowledge of the mechanism of the protection afforded by the oil and light might be obtained by the study of the amounts of phosphorus in the bones and tissues of rats which had been kept on these diets with and without cod liver oil or light, and as though it might be indicated by such a study whether the mode of protection was by a greater absorption and retention of phosphorus by the entire body or a redistribution of it so that the bones could retain a larger share.

Several studies of the ash of normal and rachitic bones have been reported (4 to 7). It has been shown that although the total ash is reduced in the rachitic bones, the normal ratio of calcium and phosphorus is not materially changed.

McClendon (8) states that the skeleton of a white rat was found to contain 82 per cent of the total phosphorus of the body and that "on the basis of two such rats the P content was about 5 mgm. per gram body weight." No information is given as to the diets or growth of these rats.

Various reports have been given of the composition of the bones in animals fed experimental diets which were poor in phosphorus or calcium (9, 10). Perlzweig (11) used diets which he considered to be poor in phosphorus, but when compared with the diets employed in our work they do not seem to be sufficiently poor to be comparable with a rickets-producing diet, which probably accounts for the only slight variations from the normal which he obtained. The low calcium diets gave figures quite comparable to those to be reported in this paper.

Heymann (12) has compared the phosphate content of the bones and tissues of rats. He does not describe the diets given and, the animals were evidently older than those used in our work. In his normal rats he obtained an average of 1.4964 gm. of P_2O_5 (0.71 gm. of P) per 100 gm. of live weight in the entire body, and 0.4408 gm. per cent of P_2O_5 (0.21 gm. of P) in the tissues. The latter figure is slightly higher than those reported for this paper.

Methods.

White rats were given the experimental diets (Table I) at the age of 4 weeks. The diets were continued for 28 to 30 days. Roentgenograms were then made and the rats killed by etherization. The gastrointestinal tract (esophagus to rectum) was re-removed and discarded, for the amounts of calcium and phosphorus contained in these organs were found to be too minute for accurate analysis. A piece of one middle rib was removed for $\frac{1}{4}$ inch on each side of the costochondral junction for histological examination. The rat was boiled in distilled water for 5 minutes, then the skeleton was dissected out. The water used for boiling was added to the soft parts. The "large bones" in the *first series* were skull and bones of the extremities, including the ilia and scapulæ but not the paws. The paws were ashed with the other small bones. In the *second series* the skull was ashed with the small bones. This was done because of the difficulty of cleaning the skull and it was felt that better bone figures could be obtained

TABLE I.*

Diet	Composition of diet.	Remarks.
N	Bread, greens, and dried milk *ad libitum.*	Gave fair growth but bones were delicate.
E M + yeast.	Egg albumin..............10.0 per cent. Butter fat................5.0 " Z 84 salt................4.1 " K_2HPO_4................1.2 " Flour...................79.2 " Yeast...................0.5 "	Contains good protein, vitamines A and B, all necessary elements in salt mixture, ample phosphorus and calcium. Gave excellent growth with normal bones.
84	Calcium lactate..........2.9 " Ferric citrate............0.1 " NaCl...................2.0 " Flour...................95.0 "	Poor growth. Diet poor in phosphorus, rich in calcium. Rickets of the Diet 84 type.
D	Egg albumin.............10.0 " Butter fat................5.0 " Z 84 salt................4.1 " Flour...................80.9 "	Contains good protein, vitamine A and probably enough B. Lacks phosphorus, rich in calcium. Fair growth, marked rickets.
84 + CLO.	Diet 84 plus 5 drops of cod liver oil every day.	This prevents rickets and improves growth.
84 + light.	Mercury vapor light through Pyrex glass.	Prevents rickets but does not improve growth.
85 C	NaCl...................2.0 per cent. Ferric citrate............0.1 " K_2HPO_4................2.9 " Flour...................95.0 "	Poor growth. Diet rich in phosphorus, poor in calcium. Rickets of the Diet 85 C type.

* For further discussion of diets see Pappenheimer, McCann, and Zucker (1).

from the other bones alone.   To the tissues (evaporated to dry-
ness) Na_2CO_3 was added to prevent possible loss of phosphorus
and to give an ash more easily handled.   This was not done to
the bones which in themselves have an excess of bases.

The parts were ashed in platinum crucibles, the ash was then
dissolved in concentrated HCl on the water bath, diluted, and
heated for 1 to 3 hours.   The phosphorus was determined by titra-
tion of ammonium phosphomolybdate.   The calcium was deter-
mined by McCrudden's method (13) gravimetrically.

DISCUSSION.

The results of the analyses are contained in Table III, and in
Table II they have been condensed to averages.   The normal
rats contained 0.52 to 0.70 gm. of phosphorus and 0.72 to 0.93 gm.
of calcium per 100 gm. of body weight.   The rachitic rats on Diet
84 contained 0.45 to 0.49 gm. of phosphorus and 0.60 to 0.81 gm.
of calcium.   Those on Diet 85 C had 0.45 to 0.49 gm. of phospho-
rus and 0.66 to 0.72 gm. of calcium, while those on Diet D had
0.38 to 0.42 gm. of phosphorus and 0.41 to 0.47 gm. of calcium.
From these data it will be seen that the rachitic rats contained
less phosphorus and calcium per 100 gm. of body weight than did
normal rats.   This was especially marked when the rats grew
fairly well as they did upon Diet D (Table I).   This diet con-
tains slightly less phosphorus than does Diet 84.   On Diet 84
rats grew poorly and contained a greater amount of calcium
and phosphorus per 100 gm. of body weight than did the rats on
Diet D, but the amounts were below those found in normals
(Table II).   The calcium figures obtained were less constant than
those for phosphorus, probably because the amounts used were
small.

The percentage of phosphorus and calcium in the ash of rachitic
and non-rachitic bones (Table III) was found to be constant,
thus agreeing with the results recorded in the literature (4 to 8).
The total ash content of the bones is reduced, but the ratio of
phosphorus and calcium remains unchanged.

In normal rats 68 to 75 per cent of the total phosphorus is in
the bones.   In rats developing rickets on Diet 84, with poor
growth, 68 to 71 per cent was found in the bones, though the
total amount per 100 gm. of rat was reduced slightly below the

TABLE II.

	All bones. Percentage of live weight.						Total percentage of live weight.						Percentage in bones.					
	P			Ca			P			Ca			P			Ca		
	Minimum	Maximum	Average	Minimum	Maximum	Average	Minimum	Maximum	Average	Minimum	Maximum	Average	Minimum	Maximum	Average	Minimum	Maximum	Average
Non-Rachitic.																		
Controls, Diets N and EM......	0.37	0.47	0.41	0.70	0.90	0.79	0.52	0.70	0.60	0.72	0.93	0.80	68	76	71	93	98	96
Cod liver oil and light prevented, Diet 84......	0.39	0.53	0.46	0.77	1.15	0.93	0.53	0.72	0.62	0.79	1.20	0.96	72	76	73	95	98	97
Rachitic.																		
Diet 84, poor growth......	0.30	0.35	0.33	0.57	0.76	0.65	0.45	0.49	0.48	0.60	0.81	0.70	67	73	69	91	95	91
Diet D, fair growth......	0.21	0.26	0.24	0.40	0.45	0.43	0.38	0.42	0.41	0.41	0.47	0.45	56	62	59	95	97	97
Diet 85 C, poor growth......	0.33	0.35	0.34	0.61	0.67	0.65	0.45	0.49	0.48	0.66	0.72	0.68	67	73	70	95	95	95

Rat No.	Sex	Diet.	Live weight.	Dry weight.	Large bones. Phosphorus. Amount. (gm)	Live weight. (per cent)	Dry weight. (per cent)	Dry large bones. (per cent)	Ash large bones. (per cent)	Calcium. Amount. (gm.)	Live weight. (per cent)	Dry weight. (per cent)	Dry large bones. (per cent)	Ash large bones. (per cent)	A Phosphorus. Amount. (gm.)	Live weight. (per cent)	Dry weight. (per cent)
929*	♀	N	65	18.2	0.172	0.26	0.94	9.6	19.4	0.350	0.54	1.92	19.4	39.4	0.281	0.43	1.5
928†	♂	N	74	19.3	0.176	0.23	0.92	10.0	19.2	0.367	0.49	1.92	21.2	41.2	0.294	0.39	1.5
927‡	♀	N	83	22.3	0.187	0.23	0.84	10.0	19.0						0.304	0.37	1.30
1315‡	♀	EM	62	18.1	0.084	0.14	0.46	8.9	19.9	0.160	0.26	0.88	17.0	38.1	0.294	0.47	1.6
1317‡	♂	EM	118	36.4	0.148	0.13	0.41	9.0		0.280	0.24	0.77	16.9		0.485	0.41	1.3
1316‡	♂	EM	141	42.1	0.167	0.12	0.40	9.2	19.4	0.320	0.23	0.76	17.6	37.2	0.517	0.37	1.2
1096§	♀	S4	39	10.1	0.087	0.22	0.86	7.3	18.0	0.184	0.47	1.82	15.5	38.0	0.129	0.33	1.2
1103§	♀	S4	41	10.0	0.092	0.22	0.92	7.3	18.2	0.209	0.51	2.09	16.6	41.0	0.139	0.34	1.3
812‖	♀	S4	45	12.1	0.094	0.21	0.97	9.3	19.2	0.180	0.40	1.49	17.8	37.3	0.134	0.30	1.1
1061•	♀	S4	46	12.2	0.105	0.23	0.86	9.1	18.1	0.180	0.39	1.47	12.0	30.8	0.144	0.31	1.1
1354**	♀	D	66	17.3	0.049	0.07	0.28	6.3		0.066	0.10	0.38	8.5		0.173	0.26	1.0
1353**	♂	D	78	20.8	0.043	0.06	0.21	4.4		0.080	0.10	0.38	8.2		0.193	0.25	0.9
1355**	♂	D	82	21.6	0.039	0.05	0.18	4.3		0.074	0.09	0.34	8.1		0.175	0.21	0.8
1404††	♂	S4+CLO.	52	13.6	0.058	0.11	0.43	7.1	19.3	0.120	0.23	0.88	14.7	40.0	0.244	0.47	1.7
1406††	♀	S4+CLO.	59	16.5	0.062	0.11	0.38	7.1	18.2	0.124	0.21	0.75	14.3	36.5	0.229	0.39	1.3
1405††	♀	S4+CLO.	60	16.8	0.064	0.11	0.38	7.9	18.8	0.126	0.21	0.75	15.6	37.1	0.234	0.39	1.3
1331††	♀	S4+light.	40	12.4	0.053	0.13	0.43	7.6	19.7	0.128	0.32	1.03	18.4	46.0	0.204	0.51	1.6
1333††	♀	S4+light.	40	12.9	0.057	0.13	0.43	7.7	18.7	0.125	0.31	0.97	17.1	42.0	0.211	0.53	1.6
1037‡‡	♂	S5 C	30	6.3	0.076	0.25	1.21	8.4	18.8	0.215	0.53	2.05	17.8	40.0	0.103	0.35	1.6
1035‡‡	♀	S5 C	35	8.6	0.084	0.24	0.97	7.5	19.3	0.164	0.47	1.91	14.6	36.9	0.116	0.33	1.3
1399§§	♀	S5 C	46	10.7	0.096	0.21	0.90	7.2	19.6	0.188	0.41	1.76	14.0	38.4	0.150	0.33	1.4

Bold faced figures denote a second series.

Rats 1331 to 1333 were taken from a series of light protection experiments of Dr. A. F. He
to be published later).

* Normal bone, delicate.
† Normal bone, thin trabeculæ.
‡ Normal bone.
§ Marked Diet S4 rickets.

	Calcium.		Tissues. Phosphorus.			Tissues. Calcium.			Total phosphorus.				Total calcium.			
	Live weight.	Dry weight.	Amount.	Live weight.	Dry weight.	Amount.	Live weight.	Dry weight.	Amount.	Live weight.	Dry weight.	In bones.	Amount.	Live weight.	Dry weight.	In bones.
	per cent	per cent	gm.	per cent	per cent	gm.	per cent	per cent	gm.	per cent	per cent	per cent	gm.	per cent	per cent	per cent
3	0.88	3.14	0.088	0.13	0.47	0.028	0.04	0.15	0.369	0.55	1.98	76	0.601	0.93	3.3	95
2	0.72	2.78	0.144	0.15	0.59	0.040	0.05	0.21	0.408	0.55	2.12	72	0.572	0.77	3.0	93
			0.146	0.18	0.68				0.450	0.54	2.10	68				
0	0.90	3.09	0.141	0.22	0.78				0.435	0.70	2.40	68				
0	0.78	2.53	0.200	0.17	0.55	0.026	0.02	0.07	0.685	0.58	1.88	71	0.946	0.80	2.6	97
2	0.70	2.36	0.232	0.16	0.55	0.025	0.02	0.06	0.739	0.52	1.76	70	1.017	0.72	2.4	98
5	0.64	2.61	0.060	0.15	0.59	0.013	0.03	0.13	0.189	0.48	1.87	68	0.278	0.71	2.7	95
6	0.76	3.16	0.057	0.14	0.57	0.015	0.04	0.15	0.196	0.48	1.87	71	0.331	0.81	3.3	95
6	0.64	2.36	0.079	0.17	0.65	0.025	0.07	0.24	0.213	0.47	1.76	68	0.311	0.69	2.6	91
2	0.57	2.17	0.070	0.15	0.57	0.015	0.03	0.12	0.214	0.47	1.76	68	0.277	0.60	2.3	95
5	0.45	1.70	0.106	0.16	0.61	0.008	0.01	0.05	0.279	0.42	1.61	62	0.303	0.46	1.8	97
5	0.45	1.70	0.131	0.17	0.63	0.012	0.02	0.06	0.324	0.42	1.56	60	0.367	0.47	1.8	97
4	0.40	1.50	0.140	0.17	0.65	0.011	0.01	0.05	0.315	0.38	1.46	56	0.335	0.41	1.6	97
8	0.86	3.30	0.078	0.15	0.57	0.016	0.03	0.12	0.322	0.62	2.36	76	0.464	0.89	3.4	97
2	0.77	2.74	0.084	0.14	0.51	0.012	0.02	0.07	0.313	0.53	1.90	73	0.464	0.79	2.8	98
2	0.77	2.75	0.090	0.15	0.54	0.012	0.02	0.07	0.324	0.54	1.93	72	0.474	0.79	2.8	98
8	1.09	3.50	0.076	0.19	0.61	0.022	0.05	0.18	0.280	0.70	2.26	73	0.460	1.15	3.7	95
1	1.15	3.58	0.079	0.19	0.61	0.019	0.05	0.15	0.290	0.72	2.24	73	0.480	1.20	3.7	96
5	0.67	3.40	0.045	0.12	0.85				0.149	0.49	2.37	69				
4	0.64	2.61	0.057	0.16	0.66	0.012	0.03	0.14	0.173	0.49	2.02	67	0.236	0.67	2.7	95
9	0.65	2.79	0.055	0.12	0.51	0.014	0.03	0.13	0.205	0.45	1.92	73	0.303	0.66	2.8	95

| Rickets (x-ray).

¶ Diet 84 rickets.

* Marked rickets.

† No rickets.

‡ Diet 85 C rickets.

§ Slight Diet 85 C rickets.

normal. The rats on Diet D, however, developing severe rickets while growing fairly well, had only 56 to 62 per cent of their total phosphorus in the bones. This percentage was lowest in the rats which grew best. Apparently in the more rapidly growing animals the soft tissues are able to take up and retain a greater proportion of the phosphorus from a very limited supply, thus reducing the share of the bones below that which is found when growth is not so rapid.

Since almost all (95 per cent) the body calcium is found in the bones in all of these animals no significant changes were found in its distribution. The total calcium per 100 gm. of rat shows a change similar to that found for the total phosphorus.

In the type of rickets produced by a diet rich in phosphorus but poor in calcium (Diet 85 C) there is no marked difference in the bone analysis from that found in the type of rickets produced by Diet 84, which was low in phosphorus and high in calcium content. Although the pathological picture of the rickets produced by Diet 85 C (1) differs somewhat from that developed on Diet 84, the phosphorus and calcium contents of the bones and of the tissues, whether calculated in percentage of live weight or of dry weight of rat, are all within the same ranges, and are definitely below the normal figures. The phosphorus which is found in the soft tissues is decreased in spite of the fact that the phosphorus content of the diet is high.

This point is of especial interest in that it tends to support the view that the two types of rickets have more in common than the somewhat diverse anatomical pictures might indicate. Moreover, the fact that the same abnormal bone composition can be produced by two diets having opposite calcium and phosphorus values would seem to indicate that this abnormal mineral content of the bones is not the result of a simple deficiency of one or the other element, but that some intermediary mechanism must come into play.

Rats on Diet 84, in which the development of rickets had been prevented by the administration of cod liver oil or by exposure to light, contain amounts of phosphorus in the entire body which are normal or even above the normal figures calculated per 100 gm. of rat (Table II). In these treated rats the bones contained 72 to 76 per cent of the total phosphorus, a figure well within the

normal range although certain of these rats grew almost as well as
the unprotected rats on Diet D, in which only 56 to 62 per cent
occurred in the bones.   There seems, therefore, to be an indication
of an increased utilization of phosphorus by the entire body in
animals, which are protected by light or by cod liver oil from de-
veloping rickets on a diet poor in phosphorus.   There is possibly,
also, an increased percentage of phosphorus in the bones.   This
is very slight, however, on the diets used, in which good growth is
not obtained.   It is unfortunate that prevention experiments
were not carried out on Diet D at this time, as the changes in
distribution should have been more marked with this diet.

SUMMARY AND CONCLUSIONS.

1. Rachitic rats contain less phosphorus and calcium per 100
gm. of body weight than do normal rats.   This is most marked
where greatest growth has occurred.

2. This reduction is the same whether the rickets has been
produced by a diet poor in phosphorus and rich in calcium or by
one poor in calcium and rich in phosphorus.

3. In rachitic rats, the bones may contain a smaller percentage
of the total phosphorus than is found in normal rats.   This
difference is not marked—except where fair growth has occurred.

4. When rickets is prevented from developing in rats given a
diet poor in phosphorus but rich in calcium by administration of
cod liver oil or by exposure to light, the total phosphorus and cal-
cium content per 100 gm. of body weight is well within the normal
range.

BIBLIOGRAPHY.

1. Pappenheimer, A. M., McCann, G. F., and Zucker, T. F., *J. Exp. Med.*,
   1922, xxxv, 421, 447.
2. Shipley, P. G., Park, E. A., McCollum, E. V., Simmonds, N., and
   Parsons, H. T., *J. Biol. Chem.*, 1920–21, xlv, 343.
3. Hess, A. F., Unger, L. J., and Pappenheimer, A. M., *Proc. Soc. Exp.
   Biol. and Med.*, 1921–22, xix, 238.
4. Gassmann, T., *Z. physiol. Chem.*, 1910–11, lxx, 161.
5. Schabad, J. A., *Mitt. Grenzgeb. Med. u. Chir.*, 1911, xxiii, 82.
6. Sherman, H. C., and Pappenheimer, A. M., *J. Exp. Med.*, 1921, xxxiv,
   189.
7. Elliott, W. E., Crichton, A., and Orr, J. B., *Brit. J. Exp. Path.*, 1922,
   iii, 10.

8. McClendon, J. F., *Am. J. Physiol.*, 1922, lxi, 373.

9. Weiske, H., *Z. Biol.*, 1874, x, 410.

10. Aron, H., and Sebauer, R., *Biochem. Z.*, 1908, viii, 1.

11. Perlzweig, W. A., *Tr. Dent. Soc. State N. Y.*, 1915, xlvii, 192.

12. Heymann, F., *Z. physiol. Chem.*, 1904, xli, 246.

13. McCrudden, F. H., *J. Biol. Chem.*, 1911–12, x, 187.

THE TRYPTOPHANE CONTENT OF SOME PROTEINS.

By CLARENCE E. MAY and EMBREE R. ROSE.

(From the Physiological Chemistry Laboratory of the University of Indiana Bloomington.)

In the present work we have devised a method for the determination of tryptophane dependent on the fact that when tryptophane is liberated by heating the protein with hydrochloric acid in the presence of Ehrlich's reagent (*p*-dimethylaminobenzaldehyde), a reaction takes place resulting in the production of an intense blue color. Although Ehrlich used a solution of very dilute sodium nitrite to aid in the production of the blue color, the present authors have not used any sodium nitrite for the development of the blue color. We have tried various methods of hydrolysis of the proteins including the pancreatic digestion method of Herzfeld,[1] hydrolysis by means of the $Ba(OH)_2$ method of Homer,[2] and hydrolysis by means of varying concentrations of acids at varying temperatures for varying times and have found what we believe to be the best conditions for the development of the color. We have taken the tryptophane content of casein as our standard and have compared the various other proteins with this standard. We calculated the tryptophane content of the various proteins on the basis that 1.5 gm. of tryptophane were yielded by the hydrolysis of 100 gm. of casein.

As results of preliminary work in the development of the method we used later for the determination of the tryptophane content of some twelve purified proteins, we established the following facts.

An intense blue color suitable for colorimetric comparisons was produced by the amount of tryptophane present in 0.1 gm. of casein hydrolyzed and diluted to a volume of 100 cc.

[1] Herzfeld, E., *Biochem. Z.*, 1913, lvi, 258.

[2] Homer, A., *J. Biol. Chem.*, 1915, xxii, 369.

The *p*-dimethylaminobenzaldehyde reagent (a 5 per cent solution of the aldehyde in 10 per cent sulfuric acid), produced more color when it was added to the digestion mixture of protein before hydrolysis had taken place. Only as little as 1.0 cc. of the reagent was necessary for the production of the maximum intensity of color when working with 0.1 gm. of dry protein.

The best conditions for hydrolysis of the protein were obtained when 50 cc. of concentrated c.p. HCl, 50 cc. of water, and 1.0 cc. of the reagent were mixed. To this mixture, weighed portions of protein (0.05 to 0.1 gm.) were added and the mixture was incubated at 35°C. for 24 hours and then allowed to stand 24 hours or longer at room temperature. This procedure gave blue-colored solutions when tryptophane-containing proteins were hydrolyzed. The solutions varied only in intensity of color. When the hydrolysis was brought about by other means, there resulted solutions of less intense blue color or solutions of a green or reddish shade.

The color produced was permanent for at least 10 days and probably for a considerably longer time.

The authors hydrolyzed thirteen proteins as follows; 0.05 gm. and 0.1 gm. portions of each protein were accurately weighed and added to separate solutions of 100 cc. of 1:1 c.p. HCl containing 1 cc. of Ehrlich's *p*-dimethylaminobenzaldehyde reagent. The digestions were at 35°C. for 24 hours followed by a standing interval for 40 hours at room temperature. The resulting solutions were all clear and possessed a clean blue color. These solutions were matched colorimetrically in a colorimeter. Table I shows the results.

Since the above work was finished, Folin and Looney[3] have published some results relative to the tryptophane content of several proteins. We did not study the same proteins that they studied. The last column in Table I shows the particular proteins we studied in common and the figures given represent the results of the work of Folin and Looney. They are given here for the sake of comparison.

[3] Folin, O., and Looney, J. M., *J. Biol. Chem.*, 1922, li, 433.

TABLE I.

Protein compared with 0.1 gm. casein standard.	Amount.	Colorimeter reading of unknown.	Colorimeter reading of 0.1 gm. casein solution.	Tryptophane content in the protein.	Tryptophane content in the protein (Folin and Looney).
	gm.	*mm.*	*mm.*	*per cent*	*per cent*
Casein..........	0.05	50	25	1.5	1.54
Lactalbumin.....	0.05	50	40	2.4	
"	0.1	50	80		
Gliadin..........	0.05	100	35	1.05	1.14
"	0.1	50	35		
Glutenin........	0.05	50	30	1.80	1.68
"	0.1	50	60		
Edestin..........	0.05	50	27	1.5	1.4
"	0.1	50	50		
Glycinin.........	0.05	50	28	1.65	
"	0.1	50	55		
Ovovitellin......	0.05	50	33	1.74	
"	0.1	50	58		
Egg albumin	0.05	100	38	1.11	1.23
" " ,....	0.1	50	37		
Phaseolin........	0.05	100	27	0.80	
"	0.1	100	53		
Maize gluten.....	0.05	100	37	1.08	
" "	0.1	50	36		
Legumin (vetch).	0.05	100	35	1.05	
" " .	0.1	50	35		
Zein.............	0.05	No color.		0.00	0.00
"	0.1	" "			
Gelatin..........	0.05	" "		0.00	0.00
"	0.1	" "			

SUMMARY.

1. Only by gentle hydrolysis of a protein can the whole of the tryptophane be obtained. With more vigorous conditions for the hydrolysis, a portion of the tryptophane is lost.

2. The best time to form the product of condensation of tryptophane and p-dimethylaminobenzaldehyde is during the liberation of the tryptophane from the protein molecule before it has a chance to be decomposed by the excess of free mineral acidity.

3. The color of the digestion mixtures varies directly as the tryptophane content of the proteins.

4. The method has a great advantage in that only small amounts of material are needed for the determinations.

YEAST AS A SOURCE OF VITAMINE B FOR THE GROWTH OF RATS.*

By CORNELIA KENNEDY AND LEROY S. PALMER.

(From the Section of Animal Nutrition, Division of Agricultural Biochemistry, University of Minnesota, St. Paul.)

Although the rations which are commonly used in the biological method of food analysis are usually referred to as purified, the fact remains that the ideal pure ration has not been attained. It is a comparatively easy matter to prepare pure casein (the protein commonly used in such rations), pure dextrin, and a chemically pure salt mixture, but when the food factors are added which are necessary for the growth and well being of an animal, the extent to which these purified constituents are being contaminated with unknown substances in many cases is not fully appreciated. Because it is necessary to add comparatively small amounts of these accessory factors in order to promote satisfactory growth, the same care has not been taken in securing them as pure from unnecessary contamination as has been done in securing pure protein, carbohydrate, and inorganic salts. For example, the factors vitamines A and B are obtained from certain animal and plant tissues and for this reason are difficult to separate from other cell constituents with which they are associated. In fact there have been few attempts to obtain either of these vitamines in the pure state.

Possibly vitamine A is more easily obtained in a condition less contaminated by adhering substances than vitamine B. In animal fats, at least, with the possible exception of cod liver oil,

217

vitamine A is associated almost entirely with substances of a known chemical character such as glycerides, sterols, phosphatides, and pigments, whose rôle in nutrition can be and has been measured. Not as much is known, however, regarding the chemical character of the substances in *extracts* from both animal and plant tissues. There is, moreover, no satisfactory standard by which to judge the purity of either vitamine, and there will not be such a standard until the nature of the vitamines themselves is understood. The amount of nitrogen present is not wholly satisfactory as a gauge of the purity of vitamine preparations, but at present is practically the only one available. In extracting vitamine B, at least, from either plant or animal tissue the majority of the extraneous substances extracted are of a nitrogenous character.

It is important in certain lines of work that a minimum amount of unknown nitrogen be added to the ration and, in fact, it is desirable in any case where the ration is in all other respects highly purified to add with the accessory food factors just as small amounts as possible of substances of unknown character. For example, if as active a preparation of vitamine B can be obtained after reducing the amount of nitrogen present by means of a special manipulation, then it is desirable to use such a preparation of the active factor with every purified ration. With this consideration in mind, we have endeavored to ascertain whether or not we could replace with yeast the wheat embryo preparation of vitamine B we have been using in our work.

For several years it has been generally considered that yeast is a very rich source of vitamine B; in fact it is more generally used, in experimental work, as a source of this factor for purified rations than any other naturally occurring food. For this reason and also because it is inexpensive and easily prepared for use, we desired to adopt it as a source of this vitamine instead of the more difficultly prepared wheat embryo extract. The results which we have obtained have been somewhat of a surprise to us inasmuch as the potency of yeast as a source of vitamine B has apparently never been questioned.

Hopkins (1), who was the first to add yeast to a purified ration, reported that the alcoholic extract of yeast added to purified rations greatly accelerated growth in young rats. Funk and Macallum (2) have shown that if 1 per cent of the ration is yeast,

young rats will grow for a short time, after which they begin to decline; but that if at least 3 per cent is added, satisfactory growth results. Drummond (3) has produced satisfactory growth in young rats when from 3 to 6 per cent of the ration consisted of dried yeast, although 6 per cent gave more satisfactory growth than 3 per cent. The same author has also shown that marmite, a commercial preparation of yeast extract used as a substitute for meat extract in the preparation of soup cubes, will produce satisfactory growth when equal to 6 per cent of the ration.

Macallum (4) found that either 2 or 6 per cent of dried brewer's yeast gave satisfactory results over short periods of time, but that for prolonged feeding experiments fresh whole yeast or autolyzed yeast liquor was necessary for the maintenance of the normal growth rate. He attributed the failure to grow on dried yeast for longer periods of time to deterioration of the growth factor through drying, for when fresh moist yeast was substituted in amounts equivalent to the amount of dried yeast used, there was an immediate resumption of growth. However, in the growth curves presented by Macallum for experiments of prolonged duration, there is seen a decided flattening of the curve at about the 100th day of the experiment, which, of course, may have been due to insufficient amounts of both vitamines B and A as no source for vitamine A^1 was provided in the ration. That the yeast is in part responsible for the flattening of the curves is shown by the fact that even the growth curves for the rats which received 18 per cent butter fat in their rations flattened considerably at about the same time.

Hawk, Fishback, and Bergeim (5) have reported satisfactory growth in young rats on rations which carried 5 per cent of the dried household compressed yeast (Fleischmann) as their sole source of vitamine B. None of these experiments extended over periods of from 10 to 13 weeks, a period in which we have shown that young rats may grow normally on a ration of low vitamine content (6). Osborne and Mendel (7) have fed small quantities of dried brewer's yeast either separately or incorporated in their

¹ That Macallum obtained remarkably good growth for a period of 100 days on a purified ration which contained lard (autoclaved for 3 hours at 120°C.) as the only fat may be explained by the fact that the casein in his ration may have carried a small amount of vitamine A.

purified food mixtures to render these mixtures as efficient for
promoting growth as those that contained their natural protein-
free milk. With 1.5 per cent of dried yeast to furnish the growth-
promoting vitamine B young rats of 50 gm. body weight grew to
maturity and sometimes produced young, although none was
reared. Later (8) they state that although rats will grow to
adult size when yeast is the sole source of vitamine B, the rats
are, with few exceptions, sterile. They also state that yeast is
not as efficient as their naturally occurring food products in pro-
moting growth.

Although no attempt has been made to give a complete résumé
of the literature bearing on the use of yeast as a source of vitamine B
for purified rations, the conclusions can be drawn that yeast is
quite generally accepted as a reliable source of the factor.

In the work which will be reported in this paper it has been our
endeavor to find out the lowest level at which yeast could be fed
to growing rats and still produce results which were as satisfactory
as those given by the use of the alcoholic extract of ether-extracted
wheat embryo. We have fed groups of rats in colonies on a basal
ration of purified casein 18 per cent, salts (9) 3.7 per cent, agar
2 per cent, butter fat 5 per cent, with dextrin to make 100 per cent,
and have supplied the vitamine B in the form of dried yeast of
various sources, both as an integral part of the ration or separately
in the form of a tablet. In reporting our results, we have taken
into consideration the amount of nitrogen added to the ration by
the yeast and also the actual weight of pure yeast that was neces-
sary to add to obtain growth. We believe that this last considera-
tion is of importance, and should be taken into account in reporting
the amount of yeast used. Unless the yeast is prepared especially
for laboratory use, it will be diluted with starch or other "filler."
Previous investigators have apparently either failed to take this
into account or have not reported their results on the basis of the
actual amount of yeast fed. The result is that it is not possible
to compare their data.

Experiment 1, Lots 1, 2, 3, 4, and 5, Chart 1.— The yeast used in Lots 1 to 4
of this experiment was a baker's yeast (Fleischmann) containing about 16
per cent yeast in the fresh condition. As soon as received at the laboratory,
the yeast was crumbled into small pieces and dried as rapidly as possible at
room temperature before an electric fan. The dried product contained

approximately 40 per cent yeast, the remaining ingredient being practically all starch, which is added by the manufacturer primarily to increase the keeping qualities of the yeast. This yeast was added to the rations to furnish pure yeast in amounts varying from 0.8 to 4 per cent (equivalent to 2 to 10 per cent of the dried product). In Lots 1 and 2, which received, respectively, 0.8 and 1.6 per cent of the pure yeast in their ration', Rat 214 was the only individual whose growth curve approximated normal. Two of the six rats in Lot 3, whose ration contained 2.4 per cent pure yeast (equivalent to 6 per cent dried product) made normal growth for 12 weeks which was the duration of the experiment. Three of the four rats in Lot 4, receiving 4 per cent of the pure yeast (equivalent to 10 per cent of dried product), made practically normal growth except for the last 4 weeks of the experiment when there was a decided flattening of their growth curve. There were no indications that the increasing concentrations of yeast in the rations of the rats of Lots 1 to 4 had the accumulative stimulating effect on the appetite which might be inferred from the reports of the results of yeast feeding to animals suffering from a complete absence of vitamine B. For example, Karr (10) and Cowgill (11) have found that yeast will restore the appetite of dogs which had been fed on diets lacking vitamine B and were in the condition of self-imposed inanition which precedes polyneuritis. Apparently one is not jusified in concluding from these results that addition of yeast to diets merely deficient in vitamine B will bring about a similar stimulation to the appetite.

Nine of the twenty-two rats in Lots 1 to 4 were females but none of these produced young during the period of the experiment.

Lot 5 was fed a dried brewer's yeast obtained fresh from a local brewery. When received at the laboratory, this yeast was in the form of a fluid containing considerable of the wort still-undergoing fermentation. It was dried to about one-fourth of its volume in a hot air current, the drying being then completed in a hot water oven. The product was of a dark brown color and had an intensely bitter taste. During the first 4 weeks of the experiment, the basal ration contained 2 per cent of this yeast, and during this period only one individual, Rat 362, made normal growth. On increasing the level of the dried yeast to 10 per cent of the ration, the growth was stimulated somewhat but at the end of 10 weeks in no case was the weight greater than one-half the normal for that age. When these rats were transferred to the rations of our breeding colony, which consists of mixed grains, meat scraps, and milk, their growth curves arose sharply toward the normal. When the same yeast was fed separately to the rats in Experiment 2 at the rate of 0.2 and 0.4 gm. per day per rat, the results were no better than when the yeast was incorporated in the ration at the level of 10 per cent. The failure of the brewer's yeast used in this experiment to promote normal growth is surprising in view of the fact that Osborne and Mendel have had remarkable success with this kind of yeast. This failure of our yeast to supply sufficient amounts of vitamine B to promote normal growth may be due either to large amounts of impurities in the yeast or to our mode of preparing it for use.

CHART 1.

CHART 1. The curves of Lots 1 to 5 inclusive represent the rate of growth of the rats which obtained their vitamine B from air-dried baker's yeast mixed in the ration in the following proportions:

Lot 1..... 2 per cent yeast equivalent to 0.8 per cent pure yeast.
" 2..... 4 "   "   "   "   " 1.6 "   "   "   "
" 3..... 6 "   "   "   "   " 2.4 "   "   "   "
" 4.....10 "   "   "   "   " 4.0 "   "   "   "

The ration of Lot 5 contained 2 per cent of dried brewer's yeast for the first 4 weeks of the experiment when the amount was increased to 10 per cent. As there was no indication that growth would be normal on this amount, the rats were taken off of the experimental feeding at the end of 10 weeks and put on our stock food with the result that the curves arose abruptly toward the normal. The rats of Lot 6 received the same basal ration as the rats of the other groups with the exception that vitamine B was derived from the alcoholic extract of wheat embryo instead of yeast. Each 100 gm. of ration carried the extract of 15 gm. of wheat embryo.

Experiment 2, Lots 7, 8, and 9, Chart 2.—The results of Experiment 1 were so unsatisfactory and unlike those of other investigators using yeast as a source of vitamine B that we decided that either the rats did not eat enough of their food to get the required amount of vitamine or else the type of yeast was at fault. We therefore modified the procedure by feeding the same yeast separately from the ration in the form of pellets. As there was no difficulty in getting the rats to eat the entire pellet, failure of the rats to grow on their allotted amount could only mean that there was not enough vitamine in the yeast to promote normal growth. For the first 4 weeks of the experiment the rats of Lot 7 were given pellets made of 0.2 gm. of dried

CHART 2. These rats derived their vitamine B from yeast pellets fed separate from the ration. For the first 4 weeks of the experiment (point x on curve) the rats Lot 7 received daily 0.2 gm. pellets of brewer's yeast. Pellets of baker's yeast weig ing 0.2 gm. were then fed up to the point marked 1 on curve, when the amount yeast was increased to 0.6 gm. The rats of Lot 8 were given pellets of brewer's yea weighing 0.4 gm. up to the point marked x on curve, when the yeast was changed baker's yeast and fed in the same amount to the point marked 1 on curve. Th amount was then increased to 0.6 gm. The rats of Lot 9 received daily from th beginning of the experiment pellets of baker's yeast weighing 0.6 gm.

brewer's yeast. Three of the five rats failed to make any growth during this period, one rat, No. 385, made about three-fourths normal growth, and another rat, No. 383, made better than normal growth. As the rats of Lot 8 were getting this same yeast at a higher level, *i.e.* 0.4 gm. daily, and were also far below the normal in their growth, both lots were changed at this point to the baker's yeast used in Experiment 1 made into pellets containing 0.08 and 0.16 gm. of pure yeast, respectively, (equivalent to 0.2 and 0.4 gm. of dried product). In Lot 7 two of the rats, Nos. 383 and 387, and in Lot 8 Rats 392 and 393 made very satisfactory growth on these amounts but as the other rats of the groups were still below normal, the amount given each group was increased (point 1 on curves) to 0.24 gm. of pure yeast (equivalent to 0.6 gm. of dried product). This increase in the amount of yeast given caused a renewal of growth so that all the rats were of practically normal weight at the end of the experiment which lasted 24 weeks. Four of the ten rats were females, and although there were some indications that one of them had young during the last week of the experiment, there was no evidence of the young obtained as she had been left in the colony and the other rats had probably destroyed them. There were no other cases of reproduction.

The rats of Lot 9 received 0.24 gm. of pure yeast (equivalent to 9.6 gm. of dried product) throughout the period of the experiment. Very good growth and some reproduction was obtained on this amount of yeast. However, the mothers destroyed their young. It is a fact worth noting that of twenty-four females used in the entire work with yeast, only five had young and these young were in no sense vigorous, normal rats. We are not prepared to say whether this absence of normal reproduction is due to a deficiency in vitamine B or to some other property of the yeast. Osborne and Mendel (7, 8) have also observed infertility in rats in whose rations yeast was the sole source of vitamine B. They do not, however, consider that lack of reproduction was caused by the yeast *per se* as two out of four rats which had been fed for a long period of time on rations containing 30 to 40 per cent yeast proved fertile when mated with vigorous females which had grown up on a normal mixed ration. It would seem, however, that the relation of yeast feeding as the sole source of vitamine B to successful reproduction and lactation would be determined only where individuals of both sexes were on a par as far as the diet was concerned. This was the case in our experiments. The successful matings in Osborne and Mendel's experiments may have been due to the high state of vigor of the females raised on the normal diets.

Chart 2 illustrates a point which we have frequently noticed in connection with our investigations on vitamines which seems to be contrary to the experience of others. That is, if the feeding work is begun with inadequate amounts of the vitamine-containing material and from time to time the amount is increased, the growth curve of the rat seldom duplicates the normal or at best only after a much longer period than when the larger amount is given from the beginning of the experiment. For example, in Lots 7 and 8, 0.08 and 0.16 gm. of pure yeast were fed to the point marked 1

on the curves where in each case the amount was increased to 0.24 gm., but with few exceptions none of these rats presented as satisfactory growth curves as those of Lot 9 to which 0.24 gm. of yeast was fed from the beginning of the experiment.

Experiment 3, Lot 10, Chart 3.—Having obtained unsatisfactory and variable results from the use of baker's yeast and brewer's yeast, we next tried growing a yeast under laboratory conditions in order to control the ingredients of the wort upon which the yeast was to grow. For this purpose we made a wort of hops and ground malt, filtered to obtain a clear medium, and inoculated the filtrate heavily with pure culture of *Saccharomyces cerevisiæ*. When active fermentation had stopped, the wort was poured on a suction filter, thoroughly washed, and then dried at room temperature before an electric fan. This yeast was made into pellets and fed to the rats in the usual way at the rate of 0.2 gm. daily. Only three rats were used in this group so as to conserve the yeast which was rather difficult to make in large amounts under laboratory conditions. These rats made a continuous slow growth but were considerably undersized at the end of 5 months. Rat 538 had two litters, the first numbering seven, four of which died soon after birth, and the remaining three were destroyed within a few days by the mother. The young of her second litter were weak, had subnormal temperature, and up to the time they were 4 weeks old had not been observed to leave the nest. At this time, two of the young rats were placed in the mother's feeding dish and ate of the ration. Almost immediately they were seized with convulsions in which they rolled around the cage seemingly in great pain and finally died, apparently from exhaustion. We can offer no explanation for these spasms, but they would seem to be in some way connected with the use of yeast in the rations of the mother rat as we have never before had this difficulty with any of our rats. Hartwell (12) has noted spasms in young rats whose mother was on a high protein ration and has ascribed the difficulty to a ration too rich in protein. Hartwell states that "It is possible that these results are due to a deficiency of some dietary factor, but this deficiency is only evident when the diet is too rich in protein. . . . A similar diet, but one with less protein, would not be deficient." McCollum, Simmonds, and Pitz (13) have reported pronounced nervous disturbances resulting in death in nursing rats whose mothers' ration contained 16 per cent casein and 15 per cent whole wheat. These investigators believed that the spasms were due to a deficiency of vitamine B, the sole source of which was the whole wheat. The protein of this ration was not over 18 per cent. We (14) have reported reproduction and successful rearing of young on a ration carrying 22.5 per cent protein derived from 18 per cent casein and 15 per cent dried egg yolk. In this ration the protein was moderately high but vitamine B was supplied in a liberal amount by the egg yolk and also by the alcoholic extract of 15 gm. of wheat embryo per 100 gm. of ration. From such results it seems probable that spasms in the nursing rat are not necessarily due to high protein but rather to a deficiency of vitamine B. The possibility remains, however, that

CHART 3. These rats derived their vitamine B from yeast pellets fed separately from the ration. The pellets given to Lot 10 contained 0.2 gm. of the yeast grown in this laboratory. The rats of Lot 11 received pellets made from distiller's yeast for the first 5 weeks of the experiment. As this yeast showed no growth-promoting properties, it was changed at this time (point marked x on chart) to 0.2 gm. pellets made of a starch-free yeast (Fleischmann) supplied in the fresh condition and dried by our usual method. The amount of yeast was increased to 0.6 gm. at the point marked 1 on the curves. In Lot 12 the pellets given the first 4 weeks of the experiment contained 0.2 gm. of a special starch-free dried yeast furnished by The Fleischmann Company. As there was an indication of a decline on this amount, the yeast was increased to 0.4 gm. daily. In this lot we did not wait for the decline to extend over several weeks as we had in the other experiments because of the difficulty of bringing the rats back to the normal rate of growth by small additions of the deficient factor. However, this increase did not stop the decline. The dotted part of the curve indicates the time during which the rats were given the feed of our stock rats.

yeast contains some other factor which is detrimental to the rearing of the young.

Experiment 4, Lot 11, Chart 3.—A dried distiller's yeast was used to furnish vitamine B for the first 4 weeks of this experiment. Pellets weighing 0.2 gm. each were given daily to the rats to the end of this period when it became apparent that normal growth could not be made on this yeast. This yeast was then replaced by the same amount of a dried starch-free yeast (Fleischmann) which was obtained fresh and dried by us in the manner previously described. This change stimulated growth to a considerable degree but at the end of 12 weeks, as the growth curve was still far from normal, the amount of yeast was increased to 0.6 gm. daily (point 1 on curves). This had practically no effect except that one female produced a litter of three, two of which died soon after birth and the other one never grew normally.

Experiment 5, Lot 12, Chart 3.—The rats of this experiment obtained their vitamine B for the first 4 weeks of the experiment from 0.2 gm. of a

TABLE I

Source of vitamine B.	Amount of product in ration.	Nitrogen content of product.	Approximate daily nitrogen intake in form of vitamine B product.	Proportion total nitrogen intake as vitamine B product.
	per cent	per cent	gm.	per cent
Dried baker's yeast.	2	5.05	0.005 –0.010	0.176–0.356
Fleischmann's yeast.	4	5.05	0.010 –0.020	0.352–0.704
" "	6	5.05	0.015 –0.030	0.526–1.05
" "	10	5.05	0.025 –0.050	0.880–1.76
Wheat embryo extract on dextrin.	11.4	0.905	0.0052–0.010	0.18 –0.352

dried, starch-free yeast obtained ready prepared from The Fleischmann Company. For the first 4 weeks of the experiment these rats, with one exception, made satisfactory growth, but there being some indications of a decline at the end of this time, the yeast was increased to 0.4 gm. daily. This amount, while it may have arrested the decline, did not bring the rate of growth back to the normal. At the end of 16 weeks all the rats were undersized but in a fairly good physical condition. There was no reproduction in this experiment. At the end of the experiment these rats were given the stock food used in our breeding colony which greatly stimulated growth except in the case of one individual, Rat 724, so that within 3 weeks time the growth curves had almost reached the normal. As the basal ration used in these experiments was known to be adequate in all respects except in vitamine B, which was furnished by the yeast, such a result would show that vitamine B was not being supplied in adequate amount.

The amount of unknown nitrogen which was actually added to the rations used is presented in Tables I and II. Column 4 of Table I necessarily

states only the approximate amount of nitrogen added, as the rats eating these rations were kept in colonies and therefore the amount of food consumed by each could be estimated only. Comparing the growth curves of Lots 1 to 5 inclusive with Lot 6 to which vitamine B was furnished by the alcoholic extract of wheat embryo, it is readily seen that yeast mixed in the ration in an amount as high as 10 per cent of the ration does not give as satisfactory growth as the same ration with the yeast replaced by the alcoholic extract of wheat embryo equivalent to 15 gm. of embryo per 100 gm. of ration. The amount of unknown nitrogen consumed daily by each rat on the yeast ration is from 0.025 to 0.050 gm., while that consumed daily by each rat on the ration carrying the embryo extract varies from 0.005 to 0.01 gm. These figures become even more significant when calculated to show the ratio of the nitrogen in the vitamine product to the total nitrogen of ration (Column 5 of Table I).

TABLE II.

Source of vitamine B.	Actual amount consumed daily.	Nitrogen content of product.	Actual amount nitrogen added to ration daily.	Proportion total nitrogen in intake as vitamine B product.
	gm.	per cent	gm.	per cent
Fleischmann's (baker's) air-dried yeast.	0.2	5.05	0.0101	0.356
	0.4	5.05	0.0202	0.711
	0.6	5.05	0.0303	1.06
Laboratory yeast.	0.2	8.55	0.0171	0.602
Fleischmann's starch-free air-dried yeast.	0.2	7.66	0.0153	0.538
	0.6	7.66	0.0459	1.61
Fleischmann's special starch-free yeast for laboratory use.	0.2	7.66	0.0153	0.538
	0.4	7.66	0.0306	1.07

In Table II the actual nitrogen intake is calculated. Referring to Lot 9, Chart 2, a group of rats that approximated normal growth, it is again readily seen that their growth curves are not as good as those of Lot 6, Chart 1. Although the amount of nitrogen actually consumed by the individuals of each group is not wholly comparable because of the difference in the method of feeding the vitamine preparations, it can, however, be safely stated that the rats of Lot 9 consumed daily more unknown nitrogen than those of Lot 6. None of the groups included in Chart 3, which received from 0.2 to 0.6 gm. of yeast per day, shows satisfactory growth. These amounts of yeast, in addition to being deficient in vitamine B, add more unknown nitrogen to the ration than the wheat embryo extract.

DISCUSSION OF RESULTS.

Our results do not support the general belief that yeast is an unusually valuable source of the growth-promoting vitamine B, or that it can be accepted as a standard product in experiments in which a vitamine B preparation is required. That its efficiency depends in part upon the manner in which it is fed is shown by the rats of Lot 8, Chart 2, to which the yeast was fed separately from the ration at the rate of 0.6 gm. daily. In this group almost normal growth was attained and some reproduction, though the young were not reared, while the growth of the rats of Lot 4, Chart 1, which received their vitamine from the same yeast but mixed in the ration in the proportion of 10 parts of yeast in 100 parts of ration was not as satisfactory. But even under our most favorable feeding conditions we have not been able to obtain as satisfactory growth as others have reported.

The reasons for these results are purely speculative. Although the type of yeast may have a bearing on its ability to absorb vitamine, this does not seem probable. Clark (15) has shown recently that the yeast cell quantitatively removes a growth-stimulating substance (bios) from wort. This may indicate the manner in which yeast derives the bulk of its vitamine B. A more reasonable view would be that the character of the wort in which the yeast is grown and the extent to which the yeast has functioned as a fermenting organism before it is removed from the wort are the important considerations in producing a yeast rich in vitamine B.[2] By analogy to higher plants it seems probable that the grow-

[2] Professor H. Maey of the Division of Dairy Husbandry kindly made a microscopic count of the total number of yeast cells per gram of yeast product with the following results:

Yeast.	Cells per gm.
Fleischmann (bakers)	3, 300, 000, 000
Laboratory	13, 200, 000, 000
Distillers	1, 100, 000, 000
Fleischmann (starch-free)	25, 760, 000, 000
" special starch-free for laboratories	11, 360, 000, 000

Since the number of cells present does not correspond to the potency of the yeast as a source of vitamine B, it strengthens the assumption that it is not the yeast *per se* which is the active agent.

ing functioning yeast cell should contain larger amounts of vitamine than the mature resting cell, or the mere budding cell.

In our opinion, the failure of our rats to reproduce normally and rear their young is one of the strongest arguments against the use of yeast as a source of vitamine B. However, we realize that this may be an arbitary standard to set as each investigator has his own standard by which to judge satisfactory nutrition. We have adopted for our general laboratory practice the McCollum standard by which satisfactory growth is measured by the attainment of normal weight and physical well being and ability to reproduce and rear young at normal intervals.

Another objection to the use of yeast as a source of vitamine B is that if it is necessary to add large amounts of yeast in order to be certain of securing satisfactory growth, it is labor lost to expend a large amount of effort in securing highly purified casein, dextrin, and salts, and then add the entire cell contents of an actively growing plant. There are also serious objections to the use of alcoholic extracts of many of the naturally occurring foodstuffs as a source of vitamine B. The leaves of plants, if cut and dried when immature; the embryos of seeds; and all glandular animal tissue contain relatively simple nitrogenous compounds which dissolve to a certain extent in alcohol. However, this unknown nitrogenous material is added in smaller amounts to a purified ration when vitamine B is furnished by extracting wheat embryo with 80 per cent alcohol than when sufficient yeast is added to the ration to furnish this factor. 10 per cent of yeast, adding to the ration from 0.025 to 0.050 gm. of nitrogen daily, does not promote growth as efficiently as 11.4 per cent embryo preparation which adds to the ration only 0.0052 to 0.01 gm. of nitrogen (Table I). When the yeast is fed separately from the ration, the amount of nitrogen added to the ration is even greater (Table II). The amount of unknown nitrogen is small in either case and may be of no special importance unless the experiment is intended to study the relative value of certain proteins or amino-acids. However, the amount of unknown material added to an otherwise carefully purified ration is greater and of a more complex nature when yeast is added than when an alcoholic extract of yeast or some other vitamine-containing foodstuff is employed.

SUMMARY.

Yeasts from different sources have been fed to experimental animals to ascertain if all yeasts were equally potent sources of vitamine B.

Two methods of feeding the yeasts were employed. In one, the yeast was mixed in a purified ration which was adequate as to all its constituents except vitamine B. In the other, the yeast was fed separately in the form of a pellet. The last method proved to be the most satisfactory.

The rats were kept under conditions favorable for reproduction, but only in a very few instances were young produced and these were never reared.

A comparison is shown between the amount of unknown nitrogen added to the ration when an adequate amount of vitamine B is fed in the form of yeast and wheat embryo extract.

A discussion of the probable reason for the failure of yeast as a source of vitamine B is given.

BIBLIOGRAPHY.

1. Hopkins, F. G., *J. Physiol.*, 1912, xliv, 425.
2. Funk, C., and Macallum, A. B., *J. Biol. Chem.*, 1915, xxiii, 413.
3. Drummond, J. C., *Biochem. J.*, 1917, xi, 255.
4. Macallum, A. B., *Tr. Roy. Canadian Inst.*, 1919, xii, 175.
5. Hawk, P. B., Fishback, H. R., and Bergeim, O., *Am. J. Physiol.*, 1919, xlviii, 211.
6. Kennedy, C., and Dutcher, R. A., *J. Biol. Chem.*, 1922, l, 339.
7. Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1917, xxxi, 149.
8. Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1919, xxxviii, 223.
9. McCollum, E. V., Simmonds, N., and Pitz, W., *J. Biol. Chem.*, 1916, xxv, 123.
10. Karr, W. G., *J. Biol. Chem.*, 1920, xliv, 255.
11. Cowgill, G. R., *Proc. Soc. Exp. Biol. and Med.*, 1920–21, xviii, 290.
12. Hartwell, G. A., *Biochem. J.*, 1922, xvi, 78.
13. McCollum, E. V., Simmonds, N., and Pitz, W., *J. Biol. Chem.*, 1916–17, xxviii, 211.
14. Palmer, L. S., and Kennedy, C., *J. Biol. Chem.*, 1921, xlvi, 559.
15. Clark, N. A., *J. Phys. Chem.*, 1922, xxvi, 42.

THE DETERMINATION OF URIC ACID.

By STANLEY R. BENEDICT.

(From the Department of Chemistry, Cornell University Medical College, New York City.)

In his article in this number of the Journal, Folin (1) presents some material which may, perhaps, be so interpreted as to confuse the subject of uric acid determination. The data given by him on the recovery of uric acid added to sheep blood are, for instance, of little service in connection with the determination of uric acid in human blood. If, as Folin reports, the blood of one species (sheep) behaves differently at different times in regard to uric acid recovery, such results would hardly furnish ground for basing a conclusion in regard to the behavior of the blood of another species. Indeed the contradictory results reported by Folin in this connection simply emphasize the fact that uric acid determination is a different problem for the blood of each species. Without quite exhaustive studies upon human blood it would seem unwise to adopt conditions such as are advocated by Pucher (2) or the exceedingly slow process of protein precipitation suggested but not urged by Folin in his present paper. There is little reason for introducing the possibility of new errors (as in Pucher's process) or of prolonging the precipitation to an interminable degree in a search for 3 or 5 per cent of uric acid which may or may not be lost when the original Folin-Wu process is applied to human blood. The errors involved here, if indeed such occur at all in human blood, are in any case of such small magnitude that they may be regarded as of no practical significance. This statement is based upon comparative analyses of more than 70 samples of human blood, a portion of each of which was precipitated by (*a*) the Folin-Wu procedure and (*b*) by heat coagulation in 0.01 N acetic acid by immersion in boiling water and final dilution to a definite volume with aluminum cream, followed by

shaking and filtration. Variation by the two methods was very
small, and while the heat coagulation yielded the slightly higher
figures, certain findings indicate a question as to whether the small
increment obtained was uric acid.   The results will be reported
in detail later only if subsequent work shall furnish more definite
information as to the nature of the substance responsible for the
small fraction of a milligram increase in the uric acid figures
obtained.   In any case I feel that the discrepancy by the two
procedures is too small to warrant a change in the method of pro-
tein precipitation from that proposed by Folin and Wu.   And
there appear to be no definite findings reported in Folin's present
paper which would lead to any different conclusion for human
blood.

In the latter portion of his paper Folin adopts, with reasonable
exactness, the present writer's technique for direct uric acid deter-
mination in blood, and urges the use of the original Folin-Denis
reagent.

Folin's implied criticism of the present writer for employing a
new reagent in connection with a radical departure in uric acid
determination may justify a word or two of explanation.
Throughout all my early work directed toward obtaining a direct
method for the determination of uric acid, using the Folin-Denis
reagent, progress was frustrated at practically every point by
the turbidities produced, and by a lack of proportionality of color
when very little uric acid was used.   Through use of the new
reagent conditions were obtained by which it was possible to
make a systematic study of the field whereby the very sensitive
direct method for uric acid determination was worked out.   It is
not especially surprising that after this technique was developed
by means of a reagent especially constructed for the purpose,
Folin is able to find similar conditions under which he can make use
of the old reagent.   There were definite reasons why I made no
special attempts to get back to the old reagent after developing the
process with one which I deem more suitable.   An enormous
amount of work would have had to be duplicated to prove that
results would be similar.   Though hundreds of analyses might
have been involved here, the crucial point was that I believed
and still believe, that the new reagent is adequate for the purpose
for which it was constructed, and that it possesses at least two

points of superiority over the old reagent. These are (a) that the new reagent is prepared with 20 minutes boiling as compared with several hours boiling necessary for the old reagent, and (b) that the new reagent has less tendency to turbidity than has the old. Folin has endeavored to meet the difficulty involved in the matter of greater tendency to turbidity by three variations from my technique; viz., the use of lithium salts, heating for a short period of a definite number of seconds, and finally by a dilution to 25 cc. instead of the 15 cc. volume which I adopted. From a practical standpoint it is obvious that these modifications rather detract from the simplicity of the process. They are also unnecessary. All my reported analyses were carried out upon bloods heavily oxalated with potassium oxalate, and I experienced no difficulty with turbid solutions. If, however, turbidity should be a troublesome factor in any laboratory, this may be readily overcome by diluting to 20 cc. instead of to 15 cc. Such dilution (of both standard and unknown) can be made either before addition of the reagents or immediately after the period of heating. While I suggested the use of sodium oxalate in place of the potassium salt, this departure is in no way necessary for the successful carrying out of the direct method.

The arsenic phosphotungstic acid reagent is easily prepared, and appears to meet the demands made upon it for both blood and urine work. After all, the ease of preparation of a reagent, and its general suitability, are considerations of more importance than its origin or its age in the literature. The Folin-Denis reagent was not the first phosphotungstic acid reagent for uric acid in the literature (cf. Moreigne, 1905 (3)), nor is it probable that any of the present reagents will be the last.

A further point in Folin's present paper requires comment. This relates to the question of the cyanide solution to be employed. Folin mentions what I have already called attention to (4); viz., that fresh cyanide solutions yield considerable blanks in the uric acid method. He further concludes that with age of the solution these reducing substances disappear, and recommends the use of a 15 per cent sodium cyanide solution which is at least 2 weeks old. I encountered these same findings, and reported that the addition of a trace of ammonia to fresh cyanide solutions reduces the blank yielded by them to practically nothing. It is

reasonable to suppose from this finding that the ammonia which forms spontaneously in cyanide solutions is responsible for the disappearance of the blank, and the addition of a little ammonia is often preferable to waiting 2 or 3 weeks. It should also be noted that 15 per cent cyanide solutions are very unstable. I began with the use of such a solution and found that figures for uric acid in blood ran consistently lower as such solutions increased in age, until results became definitely too low for the uric acid content of the blood as compared with the old method. Hence the use of a 15 per cent solution of cyanide was abandoned in favor of a 5 per cent solution to which was added a trace of ammonia. Such a solution decomposes very slowly, and yields safe and consistent results for at least 3 months or longer. Addition of sodium hydroxide to this solution is quite unnecessary.

Folin has pursued his search for a permanent standard solution of uric acid, and in this connection reports some very interesting findings concerning the uric acid-formaldehyde standard proposed several years ago by Folin and Denis. While I have not been so impressed with the necessity of a permanent uric acid standard as Folin has been, it is obvious that a solution of uric acid which can be absolutely depended upon for a period of 6 months or longer would be a very definite and valuable addition to uric acid analysis. This problem requires, however, the cooperation of many laboratories, and several years of time for its solution. Uric acid solutions appear to behave quite differently in different laboratories. Therefore, each new solution proposed must be checked up by the individual worker in his own laboratory. The standard solution which has so far probably met with most general approval is the phosphate standard proposed by Benedict and Hitchcock, which has been found to keep for at least 2 months unless subjected to very unusual conditions of temperature.

A permanent solution of uric acid might be obtained through; (a) lowering the dissociation of the acid to a negligible point, or (b) through reducing the oxygen tension of the solution to a point where oxidation of the uric acid is prevented. Spontaneous hydrolysis of uric acid at ordinary temperatures appears to be negligible, as Folin reports aqueous solutions of uric acid (containing sulfite) which have remained unaltered for at least 2 years. Folin believes that the dissociation of the uric acid-formaldehyde compound is so low in concentrated solutions that such solutions may

prove to be quite permanent in their uric acid value. Of course there is (as evidenced by the color-yielding power) very appreciable dissociation of the formaldehyde-uric acid compound even in strong solution, so that theoretically there will be some oxidation here. Whether such oxidation will be of moment can be determined only by detailed comparisons in many laboratories. Certainly Folin's new standard appears to be worthy of study.

It may be of interest to describe a uric acid solution which the present writer has been studying for 6 months, and which appears to offer promise of being reasonably permanent. In this solution oxidation of the uric acid is prevented by lowering the dissociation by means of hydrochloric acid, and by lowering the oxygen tension of the solution. Thus solutions of the full dilution required for blood analysis by the new method (0.02 mg. in 5 cc.) have been kept for 6 months with no detectable change in uric acid value. The procedure for preparation of the solution is as follows.

About 8 liters of the dilute standard solution required for the uric acid determination in blood, and containing hydrochloric acid, are prepared as described in a previous paper (4) and placed in a clean dry, narrow mouthed bottle of about 10 liters capacity. This bottle is fitted with a tight, new, clean, 2-holed rubber stopper. Through 1 hole passes a long glass tube reaching nearly to the bottom of the bottle, and which after passing out through the stopper is bent sharply so that its lower end reaches somewhat below the bottom of the bottle on the outside. The end of this tube is fitted with a short piece of clean rubber tubing and a spring clamp. Through the other hole in the stopper passes a second glass tube terminating an inch or more above the surface of the liquid in the bottle. This tube is preferably bent at right angles where it emerges through the stopper, and is also fitted with a short rubber tube and a spring clamp. A Kipp generator, charged with broken marble and hydrochloric acid, and fitted with a wash bottle is now connected with the long tube which reaches nearly to the bottom of the standard solution. Both spring clamps on the tubes to the bottle are opened, and a moderately brisk stream of carbon dioxide is passed through the solution for about 30 minutes. While the generator is still turned on, the outlet tube from the bottle is first closed with the spring clamp, so that carbon dioxide collects in the bottle under pressure. Then the inlet tube

is similarly closed and the generator turned off and disconnected.
The bottle of standard solution is now placed upon a convenient
shelf, together with the Kipp generator.   The latter is now to be
connected with the short tube which terminates above the solution,
and the generator stop-cock opened wide.   The spring clamp which
closed this tube is also opened, and left open.   Standard solution
can now be withdrawn as needed by opening the spring clamp
on the lower tube, and the volume leaving the bottle is immediately
replaced by carbon dioxide from the generator.   As the whole
system is under pressure of carbon dioxide there is no tendency for
air (oxygen) to enter it at any time.   The first few cc. of solution
withdrawn are best discarded, and the rest kept in a glass-stop-
pered bottle.   This solution can be absolutely relied upon for 2
weeks after taking it from the large bottle.

While the apparatus employed here may appear troublesome
to set up, it really requires little time or trouble, and once set up
is exceedingly convenient.   If it is desired to use the carbon
dioxide generator for another purpose it can be removed at any
time after closing the connecting tube with the spring clamp.
Of course, the generator must be connected whenever standard
solution is withdrawn, and we prefer to employ one small generator
exclusively for this purpose.   Solutions thus prepared on the 1st
of January, 1922, and kept in uncolored bottles in a warm room
near a radiator had lost no detectable quantity of uric acid on
June 20, 1922, nearly 6 months later.   What the limit of the keep-
ing quality of these solutions will be under widely varying con-
ditions can be determined only by prolonged observation in many
laboratories.   They offer a very definite advantage in requiring no
special dilution prior to use, and can be made of proper strength
for the direct determination of uric acid in urine, if desired.
Several bottles of standard solution may be connected with a
single generator by means of T-tubes, if desired.   The hydro-
chloric acid appears to prevent completely bacterial growth
indefinitely.

BIBLIOGRAPHY.

1. Folin, O., *J. Biol. Chem.*, 1922, liv, 153.
2. Pucher, G. W., *J. Biol. Chem.*, 1922, lii, 329.
3. Moreigne, H., *Ann. chim. anal. appl.*, 1905, x, 15.
4. Benedict, S. R., *J. Biol. Chem.*, 1922, li, 187.

A METHOD FOR THE PURIFICATION OF PICRIC ACID FOR CREATININE DETERMINATIONS.

By STANLEY R. BENEDICT.

(*From the Department of Chemistry, Cornell University Medical College, New York City.*)

Some years ago Folin and Doisy[1] called attention to the fact that many commercial samples of picric acid are unsuitable for use in the determination of creatinine by the micro methods, owing to the fact that such samples may yield a very considerable amount of color with alkali alone. These investigators proposed as a test for the purity of any given sample of picric acid that after addition of alkali as used in the determination of creatinine, the resulting solution of sodium picrate should not show a greater color than twice that of the picric acid solution to which no alkali had been added. Folin and Doisy also suggested a procedure for the purification of picric acid so that it would be suitable for creatinine determinations. The procedure they described was based essentially upon repeated recrystallization of the picric acid as the sodium salt.

In spite of the fact that there has been a steady demand for pure picric acid for a number of years, the situation in regard to this product has, to the mind of the present writer, been steadily growing worse. Not only have the commercial samples failed to measure up to the desired standard as often as they formerly did, but many of these samples have shown no improvement even after most arduous attempts at purification by means of the Folin-Doisy process. The procedure suggested by Halverson and Bergeim[2] for purification of picric acid in another connection also failed to improve the commercial samples to the slightest extent for purposes of creatinine determination. Indeed the situation

[1] Folin, O., and Doisy, E. A., *J. Biol. Chem.*, 1916–17, xxviii, 349.
[2] Halverson, J. O., and Bergeim, O., *J. Biol. Chem.*, 1917, xxxii, 159.

in this laboratory finally became so bad that we were compelled
either to devise a means for satisfactory purification of picric acid
or to abandon creatinine determinations.

A systematic study of the question was therefore started, and
a great number of different procedures were tried. Nearly all of
these resulted in obtaining products which were somewhat less
satisfactory than the products from which we started. It was
finally found, however, that crystallization from benzene con-
stitutes a very satisfactory method for the purification of picric
acid for creatinine determination. The procedure is ideal from
the practical standpoint, and a single crystallization from benzene
results in obtaining an excellent picric acid from the very worst
commercial sample we have ever encountered. Following is the
procedure.

It is best to start with the "technical" grade of commercial
picric acid, and there is no need for preliminary removal of the
10 per cent of water which such samples contain. 400 gm. of
the moist picric acid are placed in a 2 liter Pyrex flask and 1 liter
of pure benzene is added. The mixture is heated to vigorous
boiling on an *electric plate* with occasional shaking at first to avoid
bumping. Soon after the boiling point is reached the picric acid
practically wholly dissolves, leaving a residue of dirt and foreign
material, together with the water, which settle quickly to the
bottom when the mixture is not boiling. The hot mixture (as
nearly boiling as possible) is poured upon a large fluted filter which
has been previously moistened with benzene, the solution being
poured slowly enough so that most of the foreign sediment, to-
gether with the water, remains in the flask. This material (about
50 cc. in volume) should be discarded, and not poured upon the
filter. A hot water funnel is convenient for this filtration but can
be dispensed with if necessary. If the hot water funnel is used the
flame should be turned out just prior to beginning the filtration,
since picric acid in benzene is a highly inflammable mixture. The
clear filtrate should be received in a beaker of about 2 liters
capacity. After the filtration is completed (it should not require
more than 10 to 15 minutes) the beaker is covered with a large
watch-glass, and heated on the electric plate until the picric acid
which has begun to crystallize is again brought into solution.
The covered beaker is then allowed to stand at room temperature

for several hours or preferably over night without agitation. After this period the picric acid will be found to have crystallized on the bottom and sides of the beaker in large hard yellow-brown crystals, from which the excess of benzene can readily be drained without recourse to any filtration. The crystalline mass is washed twice by gentle rotary shaking with 75 cc. portions of benzene, and the residue finally allowed to drain thoroughly for 15 to 30 minutes. For the draining it is convenient to invert partially the beaker in a large glass funnel. The crystallized picric acid can now be freed from the last of the benzene by drying in the air (which requires a long time and is hence undesirable in the average laboratory on account of access of impurities) or better by placing in an air bath at about 80° for a few hours, with occasional stirring. The product should be finally powdered by *gentle* rubbing in a mortar, and preserved in a brown glass-stoppered bottle. About 85 per cent of the picric acid used is recovered in the purified form. The benzene may be recovered by distillation preferably *in vacuo* from a water bath.

The above process has yielded better picric acid for creatinine determinations than we have been able to obtain in any other way. The best product prepared in this way read slightly over 14 mm. by the Folin-Doisy test, with the standard set at 20 mm., which is quite the best picric acid that we have ever seen. Starting with commercial samples which read 8 to 10 mm., the single crystallization above outlined will yield a product reading from 11 to 12.5 mm., which is amply pure enough for creatinine determinations.

We are continuing the work to try to find just how free from color-yielding substances it is possible to obtain picric acid, and to attempt to identify the nature of the color-yielding material. Through concentrating the mother liquors it has been possible to obtain a product which yields a most surprising amount of color with alkali, and it is hoped that further work will reveal the nature of the substance responsible for this reaction.

THE SYNTHESIS OF WATER-SOLUBLE B BY YEAST GROWN IN SOLUTIONS OF PURIFIED NUTRIENTS.

By MARGARET B. MacDONALD.

(From the Department of Chemical Hygiene, School of Hygiene and Public Health, the Johns Hopkins University, Baltimore.)

In a previous paper (1) it was stated that yeast could be grown in a solution of purified nutrients in quantities sufficient for feeding. It was of interest to determine through feeding tests with young rats whether such yeast would serve, as do baker's and brewer's yeasts, as a source of water-soluble B. Positive results would indicate the synthesis of the vitamine by the yeast cell.

In the first test with this end in view three varieties of yeast were grown in Nutrient Solution 1 (1) and dried at room temperature. From 0.1 to 0.4 gm. of this yeast was administered daily to young rats that had been kept on a diet free from water-soluble B until they began to lose weight. The result was not decisive and a second test was undertaken in which the yeast grown in a sterile solution of mineral salts, ammonium sulfate, and cane-sugar, formed 2 per cent of the diet of young rats after a period of a water-soluble B-free diet. The observed increase in weight did not compare favorably with that reported by other investigators for commercial yeast and by Nelson, Fulmer, and Cessna (2) for yeast grown in their synthetic medium and fed at this level. The latter secured a good response, as shown by their growth curves for young rats, on the addition of 2 per cent of yeast to the basal diet. They concluded that yeast was capable of synthesizing water-soluble B when grown in a nutrient solution of the necessary mineral salts, ammonium chloride, and cane-sugar.

In view of the unsatisfactory nature of the first and second tests and the fact that the results with yeast grown in a very slightly different medium did not fully substantiate those of Nelson,

Fulmer, and Cessna, a third test was undertaken in which several varieties of yeast grown under definite conditions formed a higher percentage of the diet of young rats. One of the earlier papers of Funk and Macallum (3) shows that moderately good growth curves with 2 per cent of yeast were much improved by increasing the yeast to 6 per cent. The method for the production of the yeast and the results obtained by feeding it at 5 per cent to growing rats are set forth in this paper.

Preparation of the Yeast.

Cultures of *Saccharomyces cerevisiæ* and *Saccharomyces ellipsoideus* were obtained from Parke, Davis and Company; cultures of yeasts XII and K were furnished by the Fleischmann Laboratories and one culture was plated from a commercial yeast but not identified. These five varieties of yeast were carried through from ten to twenty successive seedings in Nutrient Solution 3 before being used for the production of the yeast in quantity.

The nutrient solution in bulk was heated to boiling on 2 successive days and was measured into sterile 1 liter Erlenmeyer flasks and heated to boiling on the 3rd day. The amount of nutrient solution measured into the production flasks was such as would give the greatest surface area, usually 150 cc. These flasks were allowed to stand for several days to test their sterility and were then inoculated from a sterile pipette with 5 cc. of a yeast suspension, made by agitating the contents of carefully inspected seeding flasks. With these precautions, possibilities of contamination were reduced to a minimum and it was necessary to discard surprisingly few flasks because of foreign growths. The production flasks were placed on shelves against the wall in a room with one window in an adjacent north wall, the yeast growing in diffused light and at room temperature. It was possible to keep the temperature of the room between 18 and 23° C. For the preparation of the seed yeast in smaller quantities a temperature of 24–30° C. was maintained. All the varieties grew at the bottom of the flasks with little or no apparent fermentation.

The length of time between inoculation and filtration of the yeast varied from 4 to 7 days, depending on the temperature, the variety of yeast, degree of aeration, nutrient solution used, and other

conditions. Usually a trial flask from the shelves was filtered on the 5th day after inoculation to serve as an index. The yeast was easily filtered by suction and washed with distilled water. The filter paper, with a layer of yeast cells about $\frac{1}{16}$ inch thick, was removed from the Buchner funnel and left on absorbent paper until the cake began to pull away from the filter paper. The yeast was then readily broken into small pieces and placed on fresh filter paper and allowed to dry in the air at room temperature for 24 hours.

The size of the seedings was obtained by filtering an aliquot part of the seeding suspension and weighing the washed yeast cells air-dry. The yield of yeast was not proportional to the size of the seeding nor to the volume of nutrient solution used. But for the five varieties of yeast grown in Nutrient Solution 3, 1 gm. of yeast, weighed air-dry, yielded from 15 to 18 gm. air-dry product. The time of production was shorter and fermentation more evident in Nutrient Solutions 4, 5, and 6, but the yield of yeast was not greater than in Nutrient Solution 3.

Nutrient Solution 3.

1 liter distilled water.
50 gm. cane-sugar (Domino).
2 " potassium dihydrogen phosphate, c.p.
2.35 " ammonium sulfate, c.p.
0.25 " calcium chloride, c.p.
0.25 " magnesium sulfate, c.p.

Nutrient Solution 4.[1]

360 gm. Porto Rican molasses substituted for 50 gm. Domino sugar in Nutrient Solution 3.

Nutrient Solution 5.

0.1 gm. Bacto-peptone (Difco) added to 1 liter of Nutrient Solution 3.

Nutrient Solution 6.[1]

A centrifuged water extract of equal weights of malt and rye, containing approximately 0.03 gm. solids per cc. of extract.

---

[1] Nutrient Solutions 4 and 6 were made by Miss M. Koch, and the samples of *Saccharomyces cerevisiæ* grown in them were prepared by her.

Basal Diet (4).

	per cent
Casein (purified)	18.0
Salt mixture 185	3.7
Agar-agar	2.0
Dextrin	71.3
Butter fat	5.0

Salt Mixture 185 (4).

	gm.
NaCl	0.173
MgSO$_4$ (anhydrous)	0.266
NaH$_2$PO$_4$.H$_2$O	0.347
K$_2$HPO$_4$	0.954
CaH$_4$(PO$_4$)$_2$.H$_2$O	0.540
Iron citrate	0.118
Calcium lactate	1.300

Feeding Test.[2]

Young rats weighing 60 to 70 gm. were placed on a basal diet adequate for growth in every respect excepting that it lacked water-soluble B (4) until they began to decline in weight. This constituted Period 1 on Charts 1, 2, and 3. During Period 2, 5 per cent of yeast replaced that amount of dextrin in the basal diet. In compounding the ration air-dry yeast was ground in a mortar and thoroughly mixed with the other ingredients.

Chart 2, Lot 3322 C shows the growth curve of a control rat that was kept on the basal diet until quite feeble. During Period 2 it received a ration in which 4 per cent of wheat embryo replaced that amount of dextrin in the basal diet.

The rats were allowed free access to a weighed quantity of food and the actual amount eaten determined by weighing what was not consumed three times a week. The food consumption was about normal. While the animals gained in weight their coats were not sleek nor was their general appearance up to standard.

DISCUSSION.

Of the many papers reporting the use of yeast as the source of water-soluble B, only a few typical ones are cited. Osborne and Wakeman (5) found that young rats declining on a diet free from water-soluble B recovered promptly on the addition of brewer's yeast extract equivalent to 0.2 gm. of yeast; and Hawk, Fishback, and Bergeim (6) secured an "immediate and pronounced increase in body weight" on the addition of 5 per cent of dried baker's yeast to the vitamine B-free diet of young rats. The growth

[2] The tests were conducted with the help and advice of Miss Nina Simmonds.

CHART 1. LOT 3320 A.
Period 1.
Basal Diet.
Casein (purified)   18.0
Salt mixture (185)   3.7
Agar-agar   2.0
Dextrin   71.3
Butter fat   5.0

Period 2.
Basal diet with 5 per cent
Saccharomyces cerevisiae grown
in Nutrient Solution 3.

LOT 3320 B.
Period 1. Basal Diet.
Period 2. Basal diet with 5 per cent
Saccharomyces cerevisiae grown in
Nutrient Solution 3.

LOT 3320 C.
Period 1. Basal Diet.
Period 2. Basal diet with 5 per cent
unidentified baker's yeast grown in
Nutrient Solution 3.

GRAMS
160
120
80
40

Lot 3320 A   Lot 3320 B   Lot 3320 C
4 Weeks

CHART 1.

CHART 2. LOT 3321 C.
Period 1.
Basal Diet.
Casein (purified)   18.0
Salt Mixture (185)   3.7
Agar-agar   2.0
Dextrin   71.3
Butter fat   5.0

Period 2.
Basal diet with 5 per cent
Saccharomyces cerevisiae grown
in Nutrient Solution 4.

LOT 3322 B.
Period 1. Basal Diet.
Period 2. Basal diet with
5 per cent yeast XII grown
in Nutrient Solution 5.

LOT 3322 C.
Period 1. Basal Diet.
Period 2. Basal diet with
4 per cent wheat embryo.

LOT 3323 C.
Period 1. Basal Diet.
Period 2. Basal diet
with 5 per cent
Saccharomyces cerevisiae
grown in Nutrient
Solution 6.

GRAMS
160
120
80
40

Lot 3321 C   Lot 3322 B   Lot 3322 C   Lot 3323 C
4 Weeks

CHART 2.

CHART 3. LOT 3321 A.
Period 1.
Basal Diet.
Casein (purified)   18.0
Salt mixture (185)   3.7
Agar-agar   2.0
Dextrin   71.3
Butter fat   5.0

Period 2.
Basal diet with 5 per cent yeast a grown
in Nutrient Solution 3.

LOT 3321 B.
Period 1. Basal Diet.
Period 2. Basal diet with 5 per cent
yeast XII grown in Nutrient Solution 3.

GRAMS
160
120
80
40

Lot 3321 A   Lot 3321 B
4 Weeks

CHART 3.

247

curves of Charts 1 to 3 compare favorably with those reported by
these investigators. More recently Kennedy and Palmer (7)
after long continued feeding of yeast have expressed doubt as
to the "suitability of yeast as a source of vitamine-B in nutrition
experiments" because of its variability.

Whether the yeast grown without vitamine would be a satis-
factory source of water-soluble B for a long period has not been
determined. For the limited time of this test the growth curves
of the rats receiving yeast grown in Nutrient Solution 3 do not
differ markedly from those of rats receiving yeast from Nutrient
Solutions 4, 5, and 6. It would seem that, in as far as its content
of water-soluble B is concerned, yeast grown in solutions of
purified nutrients is much like yeast grown in other mediums.
There can be no doubt that the rats in this test received a supply
of water-soluble B during Period 2. Those of Lots 3320 A, 3320 B,
3320 C, 3321 A, and 3321 B, received yeast grown in Nutrient
Solution 3; and the vitamine B this yeast contained was synthe-
sized by the yeast cells.

The results obtained by feeding five varieties of yeast amplify
the findings of Nelson, Fulmer, and Cessna (2) and make general
their conclusion for one variety of yeast.

BIBLIOGRAPHY.

1. MacDonald, M. B., and McCollum, E. V., J. Biol. Chem., 1920–21, xlv,
   307.
2. Nelson, V. E., Fulmer, E. I., and Cessna, R., J. Biol. Chem., 1921,
   xlvi, 77.
3. Funk, C., and Macallum, A. B., J. Biol. Chem., 1915, xxiii, 413.
4. McCollum, E. V., and Simmonds, N., J. Biol. Chem., 1918, xxxiii, 55.
5. Osborne, T. B., and Wakeman, A. J., J. Biol. Chem., 1919, xl, 383.
6. Hawk, B. P., Fishback, H. R., and Bergeim, O., Am. J. Physiol., 1919,
   xlviii, 211.
7. Kennedy, C., and Palmer, L. S., Proc. Soc. Exp. Biol. and Med., 1921-22,
   xix, 362.

STUDIES ON EXPERIMENTAL RICKETS.

XXIII. THE PRODUCTION OF RICKETS IN THE RAT BY DIETS CONSISTING ESSENTIALLY OF PURIFIED FOOD SUBSTANCES.

By E. V. McCOLLUM, NINA SIMMONDS, AND J. ERNESTINE BECKER,

(From the Department of Chemical Hygiene, School of Hygiene and Public Health, the Johns Hopkins University, Baltimore.)

AND P. G. SHIPLEY.

(From the Department of Pediatrics, the Johns Hopkins University, Baltimore.)

The experimental diets employed for the study of the etiology of rickets in this country and in England, have invariably consisted wholly or in great part of natural food substances. Since the individual foods which were employed have been studied by methods which reveal in great measure their specific dietary properties, it seemed possible to interpret satisfactorily the nature of the deficiency of these diets and hence the etiological factors operating in the causation of rickets.

However, too little is known of the science of nutrition to eliminate from the minds of critical students the possibility that when natural foods are used in feeding experiments, unappreciated factors may be active in inducing pathological states. Our own studies of the past year have revealed the existence of a distinct nutritive principle which has been confused hitherto with the vitamin A. Such results tend to discourage the acceptance of the interpretations as final which now appear as satisfactory to account for malnutrition of specific types.

In studying the etiology of rickets we have attempted to avoid error, by always employing diets with essentially like characteristics, derived from widely different kinds of natural foods, supplemented in various ways with isolated food substances. We felt that if the same histological conditions were produced in all cases

249

by experimental diets which we believed to have the same properties regardless of the sources from which the diets were derived, we were justified in drawing conclusions about the relations of the faults in the food to the structure of the bones. We hoped by this procedure to supply strong confirmatory evidence of the correctness of our conclusions, or to discover discrepancies in our results which would point the way to specific information which would extend our knowledge of the food substances which we had used.

We employed on the one hand, many diets derived from cereal grains, legume seeds, isolated proteins, inorganic salts, and fats from various sources, and on the other hand a series of diets in which liver, casein, and dextrin formed the principal components. These diets were supplemented in many ways with isolated food substances. The results presented in the earlier papers of this series of studies on rickets, rest upon observations on the bones of rats fed nearly 1,000 modifications of such diets. As our experience broadened, we found it possible to predict with certainty the changes which would occur in the skeleton as the result of restricting a young rat to a diet so constituted as to have deficiencies of certain definite kinds, no matter what materials the diet was composed of.

In order to check the accuracy of our conclusions based upon feeding diets derived principally from foods of a complex character as they occur in nature, we have extended our studies to include the effects of feeding diets consisting essentially of purified food substances. The experiments here discussed were carried out under hygienic conditions as respects temperature, cleanliness, ventilation, opportunity for exercise, and absence of direct sunlight, comparable to practically all those previously reported.

The detailed study of the influence on metabolism of modifying the amounts and proportions of the several mineral elements in the diet is extremely complex. Much time and labor must be invested in this line of study before sufficient data can be secured to make it profitable to discuss the subject in detail. It is, however, of interest to report at this time, the production of rickets in young rats restricted to a diet consisting essentially of isolated food substances.

The diets employed in the experiments here described had the following compositions:

Lot 3407.

	per cent
Wheat germ (extracted with ether and chloroform)	3.0
Wheat gluten (purified)	15.0
Gelatin*	15.0
Agar-agar	2.0
Salt Mixture XXI	3.9
CaCO₃	1.5
Dextrin (purified)	56.96
NaH₂PO₄ + H₂O	0.64
Butter fat	2.0

Lot 3408.

	per cent
Wheat germ (extracted with ether and chloroform)	3.0
Wheat gluten (purified)	5.0
Gelatin*	5.0
Casein (purified)	5.0
Agar-agar	2.0
Salt Mixture XXI	3.9
CaCO₃	1.5
Dextrin (purified)	72.6
Butter fat	2.0

* The gelatin employed was Bacto-gelatin, manufactured by The Digestive Ferments Company. It contained 0.12 per cent of phosphorus and was essentially free from other inorganic elements.

Animals on these diets showed marked signs of rickets at autopsy. On histological examination all the characteristic features of the disease were found in the bones; *viz.*, overgrowth of the cartilage, and absence of the provisional zone of calcification. There was invasion of the cartilage disc by blood vessels with a resulting irregularity of the epiphyseodiaphyseal line. The metaphysis was typically rachitic, and there was overproduction of osteoid tissue without signs of abnormal activity in resorption.

TABLE 1.

Inorganic Constituents of the Diets.

Gm. per 100 gm. of ration.

Lot No.	K	Na	Ca	Mg	S	Cl	P	Weight ratio Ca:P.	Atomic ratio Ca:P.
	gm.	gm.	gm.	gm.	gm.	gm.	gm.		
3407	0.533	0.517	1.202	0.010	0.173	0.781	0.217	1:0.181	1:0.223
3408	0.533	0.411	1.202	0.010	0.121	0.781	0.088	1:0.073	1:0.094

Composition of Salt Mixture XXI.

	gm.
$CaCO_3$	1.5
KCl	1.0
NaCl	0.5
$NaHCO_3$	0.7
$FeSO_4 + 7H_2O$	0.2

The small amount of wheat germ contained in the above rations was employed rather than an extract containing the vitamin B, because only a very incomplete separation of the vitamin from contaminating substances has been accomplished. The employment of an extract of wheat germ, would we believe, offer little or no advantage from the standpoint of interpreting the results, over the inclusion of 3 per cent of fat-free wheat germ.

The animals restricted to these diets developed xerophthalmia notwithstanding the presence of sufficient fat-soluble A to cover the minimum nutritive needs of the rat. We have described elsewhere[1] the occurrence of this "salt ophthalmia."

[1] McCollum, E. V., Simmonds, N., and Becker, J. E., *J. Biol. Chem.*, 1922, liii, 313.

STROPHANTHIN.

I. STROPHANTHIDIN.

By WALTER A. JACOBS and MICHAEL HEIDELBERGER.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

The classical studies of Fraser[1] and the confirmatory work of Feist[2] on the Kombé arrow-poison have shown it to contain a glucoside, strophanthin, which yields on hydrolysis a crystalline substance, strophanthidin, and reducing sugars. Although Fraser worked at a time when botanical classification was uncertain, it is now conceded that his material was in the main *Strophanthus kombé*. More recent comparative studies[3] have demonstrated that the glucosides of *Strophanthus kombé* and *Strophanthus hispidus* are closely related since they yield the same strophanthidin on hydrolysis. More recently Windaus and Hermanns[4] have found that the cymarin of *Apocynum cannabinum* is also a glucoside of strophanthidin (cymarigenin). Strophanthidin is thus seen to be a component of a number of glucosides which possess a characteristic action on the heart, and further study of its still obscure chemistry is of interest especially since there is suspicion of its close relationship to the still little understood members of the digitalis series.

Feist, who was the first to attempt a careful inquiry into the nature of the substance, deduced the formula $C_{27}H_{38}O_7 + 2\frac{1}{2} H_2O$. The difficulty which he experienced in preparing the anhydrous

[1] Fraser, T. R., Strophanthus hispidus; its natural history, chemistry, and pharmacology, Edinburgh, 1891. Fraser, T. R., and Dobbin, L., *Tr. Roy. Soc. Edinburgh*, 1891-93, xxxvii, 1.

[2] Feist, F., *Ber. chem. Ges.*, 1898, xxxi, 534; *Ber. chem. Ges.*, 1900, xxxiii, 2063, 2069, 2091.

[3] Heffter, A., and Sachs, F., *Biochem. Z.*, 1912, xl, 83. Brauns, D. H., and Closson, O. E., *J. Am. Pharmaceut. Assn.*, 1913, ii, 489, 604, 715.

[4] Windaus, A., and Hermanns, L., *Ber. chem. Ges.*, 1915, xlviii, 979, 991.

substance by ordinary means was overcome by the preparation of a form containing methyl alcohol of crystallization which was readily driven off. The lactone nature of the substance was determined by its behavior toward alkali. The resulting acid when liberated was not isolated as such but lost water with the formation of a new crystalline isomeric lactone which he called strophanthidinic acid lactone. The only other definite product studied by him was a crystalline strophanthic acid which resulted from the oxidation of strophanthidin with permanganate in alkaline solution and for which he adopted the incorrect formula $C_{27}H_{38}O_9$.

On the basis of analysis and titration of strophanthidin as well as data obtained from the glucoside, cymarin, itself, Windaus and Hermanns have more recently derived the formula $C_{23}H_{30}O_5$ $+ 1\frac{1}{2}$ H_2O for the air-dry substance. They likewise experienced difficulty in obtaining the anhydrous substance but since long drying *in vacuo* at 110° caused a loss of water corresponding to $1\frac{1}{2}$ mols, they concluded that it has, when anhydrous, the composition, $C_{23}H_{30}O_5$. Contrary to these workers we have experienced little difficulty in drying the substance to constant weight *in vacuo* at 110° over phosphorus pentoxide and we have noted a loss in weight corresponding to only $\frac{1}{2}$ H_2O. Similar observations have already been recorded by Brauns and Closson,[3] who, however, accepted the older formula of Feist. Our analytical data force the conclusion that anhydrous strophanthidin possesses the formula $C_{23}H_{32}O_6$ and that the loss in weight corresponding to $1\frac{1}{2}$ H_2O noted by Windaus and Hermanns was in part the result of decomposition. This conclusion is supported by the analysis of strophanthidin benzoate which these workers reported and which we have confirmed, and also by the analytical data obtained with all the derivatives to be described below. By way of confirmation of the choice between the C_{23} formula and the original C_{27} formula of Feist we believed that the preparation and complete analysis of the *p*-bromobenzoate should prove quite conclusive. The result confirmed the formula $C_{23}H_{32}O_6$.

On reduction with palladium and hydrogen strophanthidin yielded a dihydro compound, $C_{23}H_{34}O_6$, which was isolated as a mono- and a dihydrate. Although the reaction required an unusual time for completion and a relatively large amount of

colloidal palladium, it seems probable that the point of attack was a double bond. The reduction of a carbonyl group seems excluded since only a monobenzoate was obtained by benzoylation of the hydrogenated compound. Contrary to Feist and Fraser, Windaus and Hermanns state that strophanthidin reacts with ketone reagents, but they were unable to isolate definite products. We have on the contrary prepared the crystalline oxime and the phenyl and p-bromophenylhydrazones of this substance which definitely prove the presence of a carbonyl group. From all indications this is ketonic in character.

Finally, our analyses show that isostrophanthidin (isocymarigenin of Windaus and Hermanns or strophanthidinic acid lactone of Feist), like strophanthidin, possesses when anhydrous the formula $C_{23}H_{32}O_6$. In accord with this the formula of isostrophanthidin benzoate was found to be $C_{30}H_{36}O_7$.

At present it may be concluded that strophanthidin, although it may have at least one double bond, possesses mainly a saturated alicyclic skeleton. Of the six oxygen atoms, two are accounted for by the lactone group, one by a carbonyl group, and one by an alcoholic group.

EXPERIMENTAL.

Strophanthidin.—Strophanthus kombé seeds, purchased on the open market, were ground, defatted with gasoline and then exhaustively percolated with 70 per cent alcohol. The resulting extract was concentrated under diminished pressure to a thick syrup[5] which was then worked up in portions of 500 gm. In each case this amount was dissolved in 1 liter of warm water. The solution was cooled and treated with basic lead acetate solution as long as a precipitate formed. At first crude strophanthin was precipitated from the filtrate and washings with an excess of solid ammonium sulfate according to Thoms,[6] but this added purification was soon found to be unnecessary for the preparation of strophanthidin. Instead the above filtrate and washings were treated with sufficient 25 per cent sulfuric acid to precipitate the dissolved lead and then filtered. Concentrated hydrochloric

[5] The large scale operations were performed for us by Schieffelin and Co. of New York.

[6] Thoms, H., *Ber. pharm. Ges.*, 1904, xiv, 104.

acid was added to the filtrate until the latter became just acid to Congo red, an excess of acid being carefully avoided. The mixture, the volume of which was about 1,500 cc., was then heated on the water bath for 3 to 4 hours at 70–80°. The olive-colored solution slowly deposited the crude strophanthidin as lustrous plates which were somewhat tinged with green. The collected crystals were washed with water and air-dried. The yield of crude product was about 25 gm. from each kilo of seeds.

When recrystallized from 95 per cent alcohol the substance was readily purified and formed stout rhombic crystals which agreed essentially in all properties with those recorded in the literature. Its specific rotation was found to be

$$[\alpha]_D^{25} = +43.1° \ (c = 2.796 \text{ in methyl alcohol.})$$

As a rule it melted with effervescence at about 171–175° although the exact point depended upon the rate of heating. Occasionally we have obtained crystals which scarcely sintered at this point and melted at about 230°. In either case the air-dried material contained 0.5 mol of water of crystallization which it gradually lost when heated under 20 mm. pressure at 100–110° over phosphorus pentoxide, with accompanying slight discoloration. Contrary to Windaus and Hermanns[4] no further loss of water was observed on continued heating even at a pressure below 1 mm. except in cases where sulfuric acid was used as the drying agent. Here extensive decompositon occurred, as shown by the formation of a dark brown resin which no longer had the physical properties of strophanthidin. Brauns and Closson[3] likewise found no difficulty in removing the water of crystallization which they considered to be 1 molecule on the basis of Feist's[2] old formula, $C_{27}H_{38}O_7$. Our analytical data, as well as those of Brauns and Closson, obtained with anhydrous strophanthidin, agree just as well for the formula $C_{23}H_{32}O_6$ which we adopt.

Air-dry substance.

$C_{23}H_{32}O_6 \cdot \frac{1}{2} H_2O$.   Calculated.   C 66.79, H 8.05.
Found, (a).   " 66.65, " 7.91.
"   (b).   " 66.59, " 7.82.

Anhydrous substance.

$C_{23}H_{32}O_6$.   Calculated.   C 68.27, H 7.98.
Found, (a).   " 68.24, " 8.12.
"   (b).   " 68.14, " 8.18.

Strophanthidin Benzoate.—This was prepared essentially according to the method used by Windaus and Hermanns. To the properties recorded by these workers we may add that the substance is easily soluble in chloroform and acetone and sparingly so in the cold in methyl and ethyl alcohols. In concentrated sulfuric acid it gives an orange-red color which changes to a brown-orange.

Our analysis of this substance dried *in vacuo* at 110° accords with the results of Windaus and Hermanns and gives the formula $C_{30}H_{36}O_7$. The air-dry substance, however, contained $1\frac{1}{2}H_2O$ and gave

$$[\alpha]_D^{25} = + 47.8° \quad (c = 1.067 \text{ in acetone.})$$

Air-dry substance.

$C_{30}H_{36}O_7$. $1\frac{1}{2}$ H_2O. Calculated. H_2O 5 05.
                                   Found.   " 4.68.

Anhydrous substance.

$C_{30}H_{36}O_7$. Calculated. C 70.83, H 7.14.
                  Found, (a).   " 70.70, " 7.28.
                  "   (b).   " 70.66, " 7 20.

Strophanthidin p-Bromobenzoate.—2 gm. of strophanthidin were dissolved in 30 cc. of pyridine and the chilled solution was treated with 2 gm. of *p*-bromobenzoyl chloride dissolved in ether. After 24 hours the ester was precipitated with water. After digesting the collected precipitate with dilute carbonate it was filtered off and washed with water. The careful addition of water to the solution in hot acetic acid caused the separation of a rapidly crystallizing gum. The air-dried substance is sparingly soluble and contains 1 molecule of water of crystallization which is readily removed *in vacuo* at 110°. The anhydrous substance melts at 222-224° with decomposition and gives

$$[\alpha]_D^{20} = + 42.0° \quad (c = 1.094 \text{ in acetone.})$$

The analysis of this substance makes possible a definite and final decision between the formula $C_{30}H_{35}O_7Br$ based on that for strophanthidin of $C_{23}H_{32}O_6$ and the one based on the formula of Feist; *i.e.*, $C_{34}H_{41}O_8Br$.

Air-dry substance.

$C_{30}H_{35}O_7Br \cdot H_2O$.   Calculated. H_2O 2.98.

                           Found.   "   3.53.

Anhydrous substance.

$C_{30}H_{35}O_7Br$.   Calculated.   C 61.31, H 6.01, Br 13.61.

$C_{34}H_{41}O_8Br$.   "   " 62.08, " 6.29, " 12.16.

                Found, (a).   " 61.07, " 6.02, " 13.57.

                    "   (b).   " 61.25, " 6.04, " 13.73.

Strophanthidin Oxime.—The oxime was readily obtained by boiling for several hours a solution of equivalent amounts of strophanthidin and hydroxylamine hydrochloride in alcohol containing sufficient sodium acetate. Recrystallized from alcohol it forms minute, colorless, glistening prisms which melt and slowly effervesce at 270–275°. It is easily soluble in pyridine and dissolves less readily in alcohol and acetic acid. In concentrated sulfuric acid the solution is at first yellow and changes through orange to a red shade.

$$[\alpha]_D^{23} = +71.3° \ (c = 1.000 \text{ in pyridine.})$$

$C_{23}H_{33}O_5N$.   Calculated.   C 65.83, H 7.93.

                Found, (a).   " 65.95, " 7.98.

                "   (b).   " 66.12, " 7.92.

Strophanthidin Phenylhydrazone.—A solution of 2 gm. of strophanthidin in 20 cc. of absolute alcohol was treated with 1 gm. of phenylhydrazine. On allowing the mixture to evaporate spontaneously at room temperature a crop of large stout prisms formed. Recrystallized from 95 per cent alcohol the hydrazone separates as colorless, glistening, well formed prisms which sinter slightly at about 175° and melt at 230–232° to a liquid filled with bubbles. It dissolves readily in chloroform, acetone, and alcohol, and but sparingly in ether or benzene. In concentrated sulfuric acid it gives a greenish yellow color which changes through brown to red and finally to purple.

$$[\alpha]_D^{20} = -5.0° \ (c = 1.000 \text{ in chloroform.})$$

Air-dry substance (dried at 100° *in vacuo* over H_2SO_4).

$C_{29}H_{38}O_5N_2 \cdot 2 H_2O$.   Calculated. H_2O 6.79.

                         Found.   "   6.88.

Anhydrous substance.

$C_{29}H_{38}O_5N_2$.   Calculated.   C 70.42, H 7.75, N 5.67.

                Found, (a).   " 70.30, " 7.60, " 5.91.

                "   (b).   " 70.34, " 7.56, " 6.01.

Strophanthidin p-Bromophenylhydrazone.—Equimolecular amounts of strophanthidin and *p*-bromophenylhydrazine were dissolved in a small volume of acetic acid. Shortly, on rubbing, crystallization started, the hydrazone forming glistening, colorless rhombs which retained solvent of crystallization. When recrystallized from methyl alcohol the acetic acid in the compound is replaced by the alcohol, yielding stout pointed prisms which soften to a vitreous mass from 180–185° and are not completely molten until 200° is reached. The substance dissolves easily in alcohol and acetone, less readily in chloroform and ether, and very sparingly in benzene, forming a gum before dissolving in these solvents. In concentrated sulfuric acid it dissolves with a light brown color which changes first to red, then to a dirty purple, and finally becomes green.

$$[\alpha]_D^{25,5} = +105.5° \ (c = 1.004 \text{ in chloroform.})$$

Air-dry substance (dried at 100° *in vacuo* over H_2SO_4).
$C_{29}H_{37}O_5N_2Br \cdot 1\frac{1}{2}CH_3OH$. Calculated. CH_3OH 7.72.
Found.   "   7.70.

Anhydrous substance.
$C_{29}H_{37}O_5N_2Br$. Calculated. C 60.71, H 6.51, N 4.89, Br 13.93.
Found, (a). " 60.78, " 6.42, " 5.18, " 13.73.
"   (b). " 60.96, " 6.42.

Dihydrostrophanthidin.—15 gm. of carefully purified strophanthidin were dissolved in 300 cc. of pure methyl alcohol, treated with a solution of 0.5 gm. of colloidal palladium in 50 per cent methyl alcohol, and shaken in a reduction apparatus in an atmosphere of hydrogen. Absorption of the gas, which occurred at the rate of 20 cc. for the 1st hour, rapidly decreased to a rate of 5 to 8 cc. per hour. After 3 days, an additional 0.5 gm. of colloidal palladium was added. This caused, however, only a temporary acceleration of the hydrogen absorption. After 2 weeks this had practically ceased, a total of 945 cc. having been absorbed, an amount which is not far removed from 1 mol, especially where leakage over so long a period was unavoidable. The solution was acidified with a few drops of acetic acid and the coagulated palladium was filtered off. After boiling off the alcohol from the filtrate a mixture of oil and water remained which was boiled with 1 liter of water and a little bone-black. The filtered solution formed an emulsion which slowly crystallized when allowed to

cool and be rubbed. After collecting, washing with water, and drying, the yield was 12 gm. Recrystallized from a small volume of 50 per cent alcohol, the substance separates as a dihydrate in the form of glistening rhombic prisms. It melts and effervesces at 100–103°, then resolidifies and again melts with effervescence at 145–147° or merely sinters at this point and then melts at about 190–195°, depending upon the rate of heating. It dissolves readily in alcohol, acetone, and acetic acid, not so easily in chloroform, hot water, and hot benzene, and but sparingly in ether. In methyl alcohol

$$[\alpha]_D^{23} = +34.85° \quad (c = 1.004).$$

In concentrated sulfuric acid dihydrostrophanthidin gives a bright red-orange color of a much brighter, clearer, and permanent tint than that given by strophanthidin. The latter gives at first a dirty orange with a greenish tinge, later becoming a deep brown-red. Although it retains its water of crystallization even after long exposure to air, the substance loses this completely in a few hours at room temperature in a vacuum desiccator over sulfuric acid. The resulting product melts at 190–195° and is anhydrous since it loses but little more when heated *in vacuo* at 110° over phosphorus pentoxide.

Air-dry substance (dried at first at room temperature and finally at 100° *in vacuo* over H_2SO_4).

$C_{23}H_{34}O_6 \cdot 2H_2O.$ Calculated. H_2O 8.14.
Found. " 8.17.

Anhydrous substance.

$C_{23}H_{34}O_6.$ Calculated. C 67.99, H 8.44.
Found. " 68.20, " 8.43.

A different hydrate was also obtained by maintaining an aqueous solution near the boiling point for some time with occasional rubbing. Characteristic rhombs were formed containing 1 molecule of water of crystallization. These were dissolved in a small volume of warm 50 per cent alcohol, treated with hot water to turbidity, and seeded. This form, when rapidly heated, melts and effervesces at 145–147°, and then resolidifies. If the rate of heating is too slow it merely sinters at this point and melts at 190–195°. In solubilities it resembles the dihydrate with the exception of its lower solubility in hot water.

Air-dry substance (dried at 100° *in vacuo* over H_2SO_4).

$$C_{23}H_{34}O_6 \cdot H_2O. \quad \text{Calculated.} \quad H_2O \ 4.24.$$
$$\text{Found.} \qquad \text{`` } 4.01.$$

Anhydrous substance.

$$C_{23}H_{34}O_6. \quad \text{Calculated.} \quad C \ 67.99, \ H \ 8.44.$$
$$\text{Found.} \qquad \text{`` } 68.02, \text{ `` } 8.37.$$

Dihydrostrophanthidin Benzoate.—This substance was prepared like the corresponding strophanthidin derivative. Recrystallized from 85 per cent alcohol it forms minute glistening prisms which melt with effervescence at 225–227° and are soluble in chloroform and acetic acid and less readily so in alcohol and benzene.

$$C_{30}H_{38}O_7. \quad \text{Calculated.} \quad C \ 70.55, \ H \ 7.50.$$
$$\text{Found, (a).} \quad \text{`` } 70.70, \text{ `` } 7.54.$$
$$\text{`` \quad (b).} \quad \text{`` } 70.73, \text{ `` } 7.29.$$

Isostrophanthidin.—(Strophanthidinic acid lactone of Feist, isocymarigenin of Windaus and Hermanns.) This was prepared in about 50 per cent yield by a method which was essentially that used by Windaus and Hermanns for the preparation of isocymarigenin. The air-dry material contains water, which, contrary to these workers, is readily removed on drying the substance *in vacuo* at 100° over phosphorus pentoxide. The analytical data indicate that the air-dry material possesses the same formula as strophanthidin, $C_{23}H_{32}O_6 \cdot \frac{1}{2} H_2O$.

$$[\alpha]_D^{21} = +36.2° \ (c = 0.705 \text{ in methyl alcohol.})$$

Air-dry substance.

$$C_{23}H_{32}O_6 \cdot \tfrac{1}{2}H_2O. \quad \text{Calculated.} \quad H_2O \ 2.18.$$
$$\text{Found.} \qquad \text{`` } 1.78.$$

Anhydrous substance.

$$C_{23}H_{32}O_6. \quad \text{Calculated.} \quad C \ 68.27, \ H \ 7.98.$$
$$\text{Found, (a).} \quad \text{`` } 68.49, \text{ `` } 8.04.$$
$$\text{`` \quad (b).} \quad \text{`` } 68.75, \text{ `` } 7.88.$$

Isostrophanthidin Benzoate.—Isostrophanthidin was benzoylated as in previous cases. When recrystallized from alcohol, in which it is very sparingly soluble, the benzoyl derivative forms rosettes of microscopic leaflets which melt with preliminary sintering at about 270° when rapidly heated. It is soluble in chloroform and acetone, less readily in acetic acid.

$$[\alpha]_D^{23} = +38.0° \ (c = 1.002 \text{ in chloroform.})$$

$$C_{30}H_{36}O_7. \quad \text{Calculated.} \quad C \ 70.83, \ H \ 7.14.$$
$$\text{Found.} \qquad \text{`` } 70.98, \text{ `` } 7.08.$$

THE DETERMINATION OF THE TITRATABLE ALKALI OF THE BLOOD.

By ISIDOR GREENWALD and GERTRUDE LEWMAN.

(From the Harriman Research Laboratories, Roosevelt Hospital, New York.)

INTRODUCTION.

That the reaction of the blood is alkaline and that, as a result, a considerable quantity of acid may be added to it before it will show an acid reaction to litmus or to most of the common indicators has been known for a long time. That a measure of such titratable alkalinity was desirable was immediately apparent and several attempts were made to devise methods for this purpose.[1] The simplest of these involved only the addition of standard acid to the diluted blood until an acid reaction to the indicator test paper was obtained. In various modifications of this method, plasma or serum was used instead of blood, provision was made for removal of carbon dioxide or the proteins were precipitated by the use of neutral salts or of alcohol. These protein precipitates always included various ions, not in exact equivalence, the loss of which from the solution affected the value of the titration. When the proteins were not removed, their buffer action obscured the end-point.

That the carbon dioxide content of the blood or plasma could be used as a measure of the blood alkali was soon recognized and several attempts were made to devise suitable methods for this determination, either directly in the blood or plasma or, indirectly by determining the carbon dioxide content of the alveolar air which is in equilibrium with arterial or venous blood. Since the method of Van Slyke and Cullen for the determination of the carbon dioxide-combining power of the plasma was published,

---

[1] For references to the older literature see Van Slyke and Cullen (1).

263

its simplicity and accuracy have brought it into general use and it has not only practically supplanted all other methods but has made possible both clinical and experimental observations for which no other method could have been employed.

Nevertheless, the method is not free from objections. To obtain accurate results, the blood must be collected without stasis and the plasma must be separated without loss of carbon dioxide. Both of these conditions are often difficult to realize and, frequently, no serious attempt is made to do so. It often happens that the blood is almost completely reduced when collected and is then received and centrifuged without precautions to prevent the escape of carbon dioxide. The errors resulting tend to neutralize each other but the exact resultant, in any given case, cannot be definitely known. After separation, the plasma is saturated with the operator's alveolar air or with a mixture of air and carbon dioxide containing the latter at 40 mm. tension. If the alveolar CO_2 tension of the subject is not 40 mm., or the same as that of the operator, another error is introduced as was pointed out by Joffe and Poulton (2) and by Peters and Barr (3).

This last objection is obviated by the use of Van Slyke's later methods (4). In one of these, the bicarbonate content is calculated from the total CO_2 content and the pH determined by Cullen's colorimetric method (5) by the use of the formula $BHCO_3 = $ (total CO_2) $\dfrac{10^{\,pH - pK_1}}{10^{\,pH - pK_1} + 1}$ which is derived from Hasselbalch's equation $pH = pK + \log \dfrac{BHCO_3}{CO_2}$. In the other method, after addition of an excess of acid to the plasma and removal of the CO_2, the diluted, acidified plasma is titrated back to the reaction of another sample of plasma that has only been diluted. But to use either of these methods it is essential that the blood be collected absolutely without stasis and that the plasma be separated with absolutely no loss of CO_2. These requisites are probably not realizable under clinical conditions.

The determination of the CO_2 capacity of the blood at a definite CO_2 tension, as employed by Henderson and Morriss (6), has not come into general use; perhaps because the procedure is not so simple as that for a similar determination in the plasma.

Principles upon Which Proposed Method Is Based.

The cations of the blood, represented by B, are combined with anions such as 'Cl, 'SO$_4$, 'H$_2$PO$_4$, proteins, lipoids (?), and 'HCO$_3$. If a suitable acid precipitant (HA) be added, the proteins are precipitated and combined with the acid precipitant, the lipoids are carried down with this precipitate and in the filtrate are found BCl, BH$_2$PO$_4$, BA, and excess HA. The equations may be written

$$BHCO_3 + HA = BA + H_2CO_3$$
$$B_2HPO_4 + HA = BH_2PO_4 + BA$$

$$Protein \Big\langle {COOB \atop NH_2} + 2HA = Protein \Big\langle {COOH \atop NH_2HA} + BA$$

If now, the excess of free acid (HA) in the filtrate be determined by titration with standard alkali and a suitable indicator and then the total amount of precipitating anion, free and combined, in the same filtrate be determined by a suitable method, the difference will represent the amount of (A) in the filtrate that is combined with (B) derived from the blood. By a suitable choice of indicators, the amount of buffers (phosphoric acid, etc.) in the filtrate may be estimated.

Since the desired value is the difference between the two quantities actually determined, it is obvious that these should be kept low in order that the sum of the errors inherent in their determination should not be too large a fraction of the difference.

Of all the protein precipitants tried, only picric acid appears to fulfill the requirements. Only a small excess is required and this is readily titrated with 0.01 N NaOH and even with 0.005 N NaOH with methyl red as indicator, or with the former to neutral red, phenol red, or thymolphthalein. The total of free and combined picric acid can be determined gravimetrically as nitron picrate (7) or volumetrically by tritration with titanous chloride (8, 9). Attempts at a colorimetric method depending upon the color of the picric acid, of its reduction products, or of isopurpuric acid were all unsuccessful. Titration with night blue (10) was also unsatisfactory, for it was found that the composition of the night blue picrate varied with the conditions of the precipitation.

Towards methyl red, picric acid behaves strictly as a mono-basic acid, the calculated value being obtained on titration with NaOH. But as the reaction becomes more alkaline, increasing amounts of alkali are bound so that the alkali value of the picric acid must be determined by experiment. It varies somewhat with the concentration and possibly with the presence of other salts so that the final results with indicators having a color change above pH 6.0 are not quite so accurate as those obtained with methyl red.

Usefulness of the Different Indicators.

The different indicators may be used to serve different purposes. Methyl red gives the most closely agreeing duplicate determinations and can be used with as little as the equivalent of 0.3 cc. of blood. However, the end-point, pH 6.0, is outside the physiological range. As nearly as can be determined with the aid of phosphate solutions and of sodium picrate, the end-point shown by phenol red is very nearly pH 7.4, the reaction of normal blood. The phosphoric acid and other buffers are then most nearly in the condition in which they exist in the blood. The blue color of brom-thymol blue also appears at about pH 7.4 but the change is no sharper than that with phenol red and it cannot be followed by the use of thymolphthalein in the same sample. Neutral red was used early in the work but was discarded as offering no advantages over phenol red.

The value obtained with the use of thymolphthalein changes more rapidly as acid is injected, *in vivo*, than does that obtained with either of the other indicators and, therefore, may prove to be of greater clinical value as an indicator of acidosis, although the end-point is outside the physiological range.

Directions.

(a). Gravimetric Method, Using Nitron.

If available, 5 cc. of the oxalated blood are measured into a 50 cc. volumetric flask and are diluted with about 10 cc. of water. About 30 cc. of 1 per cent picric acid solution are then added, with shaking, and the mixture is diluted with water to the mark. The mixture may be filtered in 30 minutes, or, if

shaken frequently, in 10 minutes or less. 30 cc. of the filtrate are heated to boiling in a 150 cc. Erlenmeyer flask, cooled in water, and then titrated with 0.01 N NaOH, using 4 drops of 0.01 per cent methyl red as indicator. After the end-point has been reached, 4 drops of 0.01 per cent phenol red are added and a second titration is made to the appearance of a definite orange color. Finally, 8 drops of 1 per cent thymolphthalein may be added and a third titration obtained. Daylight or light from a "Daylite lamp" should be used. The liquid is then strongly acidified with acetic acid, heated to boiling, and the picric acid is precipitated by the addition, from a dropping tube, of a 1 per cent solution of nitron in 10 per cent acetic acid. The precipitate is filtered out on a tared Gooch crucible, is washed with water, and is then dried at 110° for 1 hour, cooled, and weighed. If thymolphthalein has been employed, a correction of 0.1 mg. for each drop of 1 per cent solution used is subtracted to allow for the amount of thymolphthalein carried down with the precipitate. The molecular weight of the nitron picrate is 541. Theoretically, if each molecule of picric acid is neutralized by 1 molecule of NaOH 1 mg. of nitron picrate = 0.1848 cc. of 0.01 N NaOH. This is the value actually obtained by experiment with methyl red. As has already been explained, other factors must be used with other indicators. These factors are best obtained by titrating 5 cc. portions of 1 per cent picric acid with 0.01 N NaOH, using all three indicators in turn, then precipitating with nitron, weighing the nitron picrate, and calculating its value in terms of 0.01 N NaOH. The values found in the course of this work were 1 mg. of nitron picrate = 0.1890 cc. of 0.01 N NaOH to phenol red and 0.1958 cc. to thymolphthalein.

From the alkali equivalent of the nitron picrate, there is deducted the amount of alkali required to neutralize, to the corresponding indicator, the free acid in the sample. The difference represents the amount of alkali previously combined with protein or with carbonic acid in the blood. The results are then calculated in terms of cc. of 0.1 N NaOH per 100 cc. of blood by dividing by 3 and multiplying by 10. The error is within ± 1 per cent with methyl red and is less than ± 2 per cent with the other indicators.

If so much as 5 cc. of blood is not available, satisfactory determinations may be obtained with 1.6 cc. of blood. This is

measured into a 16 cc. volumetric flask with pointed bottom, made so that it will fit into a metal centrifuge tube holder, where it is diluted with water and precipitated with from 9 to 10 cc. of 1 per cent picric acid. After shaking and standing, the flask is centrifuged and the supernatant liquid is filtered through a small paper. 10 cc. of the filtrate are taken for a determination, which is carried out as already described except that a smaller flask and smaller amounts of indicators are used. The error is less than $\pm$ 2 per cent with methyl red and less than $\pm$ 3 per cent with the other indicators.

(b). *Volumetric Method, Using Titanous Chloride* (8, 9).

If a volumetric method for the determination of the picric acid is desired, or if only a very small amount of blood is available, titration with titanous chloride may be used. The determination is then carried out in less time than with nitron, for the hour's drying at 110° and the subsequent cooling are avoided but it requires more care in the preparation of the reagents and in the determination.

From 0.3 to 0.5 cc. of blood in a 0.5 cc. Mohr pipette graduated to the tip at intervals of 0.01 cc. and capable of being read to 0.005 cc. are measured into a 5 cc. centrifuge tube, graduated in 0.05 cc., and containing a few drops of water. The pipette is rinsed with this water and then with a little fresh water. Picric acid solution is then added to make the final volume ten times that of the blood taken, the tube is covered with a small rubber cap, shaken, allowed to stand, and then centrifuged. As much of the supernatant liquid as can be obtained is pipetted off with a 3 cc. Mohr pipette, graduated to the tip in 0.025 cc. and delivered into a conical flask, 14 cm. high, 30 mm. in diameter at the bottom and with a neck 18 mm. in diameter, in which the free acid is titrated, using only methyl red (1 drop) with 0.005 N NaOH from a 2 cc. burette, graduated in 0.01 cc. (A few trials with other indicators were not very satisfactory, others may be more successful in observing the color changes.) Add 20 drops concentrated H_2SO_4 and insert a rubber stopper carrying an inlet tube which reaches nearly to the bottom of the flask, an exit tube, and a glass plug. While passing a stream of carbon dioxide from

a generator or from a tank, heat to boiling, remove the glass plug and through this opening run in from a 10 cc. burette 10 cc. of approximately 0.05 N titanous chloride solution. Replace the plug and continue the boiling for 5 minutes. The solution should retain a pink color, indicating the presence of an excess of titanous chloride. The flask is then cooled in water, the plug is removed, and the excess of titanous chloride is titrated with an approximately 0.05 N solution of ferric ammonium sulfate. When the titration is nearly completed, one-fifteenth volume of 10 per cent NH_4CNS is added and the titration continued to the appearance of a red color. The stream of carbon dioxide is not interrupted until the titration has been completed. The excess of titanous chloride should require at least 2 cc. of ferric ammonium sulfate solution. All three solutions are kept free from air in bottles of the design shown in Fig. 1. The ferric ammonium sulfate solution keeps well and need be standardized against picric acid only once. The titanous chloride deteriorates slowly and should be checked daily against the ferric ammonium sulfate. Each millimol of picric acid requires 18 millimols of titanous chloride. 1 cc. of the 0.05 N titanous chloride (or ferric ammonium sulfate) is, therefore, equivalent to $\frac{1}{360}$ millimol of picric acid or to $\frac{1}{1.8}$ cc. of 0.005 N alkali. The amount of alkali required to titrate the free acid is subtracted from the alkali equivalent of the titanous chloride used and the difference is multiplied by the appropriate factor to obtain the result in terms of cc. of 0.1 N alkali per 100 cc. of blood. The results may vary from a control by the nitron method, in which 30 cc. of filtrate or the equivalent of 3 cc. of blood are used, by as much as 4 per cent (very seldom more) though they are generally somewhat closer.

Titration with titanous chloride may also be employed with larger quantities of picric acid filtrate and three indicators, but the results, while more accurate than in the micro method, are not quite so satisfactory as are those obtained with the use of precipitation with nitron.

The micro method was developed with the intention of applying it to such quantities of blood as might readily be obtained by pricking a finger. It was found, however, that considerable pressure was necessary in order to obtain 0.3 to 0.5 cc. of blood. The results obtained with samples taken from different fingers differed

considerably and were almost all greater than those obtained by the nitron method on a sample of blood taken from a vein immediately thereafter. Apparently, pressure mixed sufficient tissue juice of a high titratable alkalinity (to methyl red) with the blood to invalidate the results.

Moreover, each of the three subjects used for this experiment (Table X) gave it as his opinion that the puncture of the vein involved less inconvenience than the collection of 0.5, or even 0.3 cc. of blood from a finger. It certainly is easier for the operator. It is, therefore, recommended that at least 1.6 cc. of blood be made available and that the micro method be used only whenever, as in experiments upon small animals, not even so much as 1.6 cc. of blood are available.

There is a source of error that is, apparently, insignificant with normal blood but which may be of importance with some pathological bloods. Titanous chloride is a very powerful reducing substance, even reducing, under the conditions of the determinations, a little of the oxalate used as an anticoagulant.[2] It is possible that some bloods should contain some reducible substance (glucose is apparently not affected[2]) the presence of which would make the values for the titratable alkali too great.

DISCUSSION.

The results summarized in Tables I to IV, inclusive, show that the values obtained by this method are not affected by variations in the amount of picric acid used for the precipitation of the proteins, by extreme variations in the carbon dioxide content of the blood, by the presence of a fair excess of oxalate nor, to any considerable extent, by standing. That sulfuric and lactic acids and sodium bicarbonate, added *in vitro* are quantitatively recovered is shown by the figures in Table V.

The experiments with intravenous injections of acid show a very prompt diminution in the titratable alkali as acid is injected. Since this is, at first, almost entirely at the expense of the bicarbonate of the blood, it is not surprising to find that the values for the CO_2 content and CO_2-combining power of the plasma fall more rapidly, at first, than do those for the titratable alkali.

[2] Unpublished observations.

But as the bicarbonate reserve is exhausted and as the condition of the animal becomes more critical, the diminution in the titratable alkali becomes more pronounced so that when the CO_2 content and CO_2-combining power have fallen to about one-half their normal values, the titration to methyl red has diminished almost as much and that to thymolphthalein even more.

The figures in Table VIII indicate that a similar relation exists in human blood. Thus, the seven normal bloods, having an

TABLE I.

Effect of Varying Concentrations of Picric Acid on the Determination of the Titratable Alkali of Sheep Blood.

0.1 N alkali per 100 cc. of blood.

1 per cent picric acid.	Methyl red. pH = 6.0	Neutral red. pH = 7.0	Phenol red. pH = 7.4	Cresol red. pH = 7.8	Thymolphthalein. pH = 9.0
vol.	cc.	cc.	cc.	cc.	cc.
5	49.6	49.5	46.5	44.4	40.0
6	49.4	49.7	46.0	44.4	41.3
8	50.7	50.6	45.9	44.2	41.9
6	45.9	45.2	43.1	43.6	37.3
7	45.8	44.9	43.2	43.2	37.7
6	46.3	46.0			39.6
8	46.0	45.7			37.4

Three different samples of oxalated sheep blood were treated with the number of volumes of 1 per cent picric acid shown in the first column and the mixtures were then diluted with water to ten times the volume of blood taken. 30 cc. of the filtrate, equivalent to 3 cc. of blood, were titrated in succession with 0.01 N NaOH, using the indicators in the order given. (Phenol and cresol red require two different samples, otherwise the entire series can be run on one sample.) The liquids were then treated with acetic acid and nitron and the nitron picrate was filtered out and weighed. It is evident that varying the amounts of picric acid used does not affect the result. When 8 volumes of picric acid are used, the excess in the filtrate is large and even slight errors in the titration or in the weighing of the nitron picrate introduce relatively large errors into the desired quantity, the difference. Therefore, it is advisable not to use more than 6 or 6.5 volumes of picric acid and with bloods that are known to be poor in protein (cells) to use only 5 volumes or even less. The buffer property of picric acid (p. 272) is probably responsible for the greater deviations observed as the pH of the indicator change rises above 6.0.

TABLE II.

Titratable Alkali of Aerated Blood and of the Same Blood Saturated with CO_2.

0.1 N alkali per 100 cc. of blood.

Blood.	Methyl red.	Neutral red.	Phenol red.	Cresol red.	Thymol-phthalein.
	cc.	cc.	cc.	cc.	cc.
Aerated...............	49.5	49.7	45.8	44.0	40.4
Saturated CO_2.........	48.7	48.5	45.5	44.0	40.1
Aerated...............	40.7				
Saturated CO_2.........	40.9				
Aerated...............	40.1				
Saturated CO_2.........	39.9				

The oxalated sheep blood was divided into two parts. One was aerated by shaking small quantities with air in large flasks, the other was saturated with CO_2 by passing the gas from a tank through water and then through the blood. Samples of each were then taken and analyzed in the usual manner.

TABLE III.

Effect of Oxalate on Titratable Alkali.

0.1 N alkali per 100 cc. of blood.

Blood.	Methyl red.	Phenol red.	Thymol-phthalein.
	cc.	cc.	cc.
Defibrinated sheep blood	45.8	43.1	37.5
" " " +0.4 per cent $Na_2C_2O_4$.	46.4	43.6	38.3
" " "	46.2	45.2	39.6
" " " +0.4 per cent $K_2C_2O_4$..	47.0	44.7	38.8
" " " +1.0 " " $Na_2C_2O_4$..	47.3	44.6	

Only 0.1 per cent oxalate is needed to prevent coagulation. Quantities four times, and even ten times, so great exert only a slight influence on the titration. However, a large excess should be avoided for it increases the amount of picric acid required for complete precipitation of the proteins and that is undesirable (p. 277) and, in the volumetric method, some of the oxalate is reduced by the titanous chloride, which makes the final results too high.

average plasma CO_2-combining power of 61 volumes per cent had an average titratable alkali content equivalent to 44.8 cc. of 0.1 N alkali per 100 cc. of blood to methyl red, 37.9 cc. (only 6) to phenol red and 32.3 cc. (only 4) to thymolphthalein.   With the exception of the one from a severe case of nephritis, the samples of blood the plasma of which had a CO_2-combining power of from 15 to 25 volumes per cent had a titratable alkali content of about 20 cc. to

TABLE IV.

Effect of Standing on the Titratable Alkali of the Blood.

0.1 N alkali per 100 cc. of blood.

Blood.	Temperature.	Time.	Methyl red.	Phenol red.
		min.	*cc.*	*cc.*
Human.	Room.	10	44.3	38.6
	"	40	44.2	38.8
	"	70	44.7	39.2
	"	100	43.2	38.2
	"	160	43.0	38.2
	"	220	43.6	38.9
		hrs.		
Human.	"	1	45.3	43.8
	Ice box.	17	43.2	40.1
	" "	41	42.5	39.5
	" "	65	41.5	38.5.
	" "	89	40.4	36.0
Sheep.	30°C.	1	49.6	46.1
	Room.	4	49.4	46.5
	+Ice box.	20	49.5	45.8

In these samples, the rate of acid production was very low. In the human bloods, this may have been due to the fact that they were nearly completely deoxygenated. Other samples might have shown a greater production of acid (11,12).

methyl red, 15 cc. to phenol red, and 3 cc. to thymolphthalein.   In other words, a reduction to 0.33 or 0.25 of the normal value of CO_2-combining power of the plasma was accompanied by a lowering of the titratable alkali of the blood to about 0.5 with methyl red, to 0.4 with phenol red, and to as little as 0.1 of the normal with thymolphthalein. The titration to thymolphthalein may, therefore, be the most valuable, clinically in spite of the slightly

greater error inherent in its determination. The one exception
to this marked lowering of the titration to thymolphthalein was
observed in a sample of blood from a patient with advanced
nephritis, taken shortly before death. The non-protein nitrogen
was 263 mg. per 100 cc. and the cell volume was only 18 per cent.

TABLE V.

Effect on Titratable Alkali of Blood of Acid and Alkali Added in Vitro.

0.1 N alkali per 100 cc. of blood.

Blood.	Methyl red.	Neutral red.	Phenol red.	Thymolphthalein.
	cc.	cc.	cc.	cc.
Human (3 days old) + 2 cc. 0.8 per cent NaCl.	43.0	38.3		27.7
" (3 " ") + 2 " 1.01 N H_2SO_4.	23.4	19.7		6.7
" (3 " ") + 2 " 1.10 N lactic acid.	24.2	19.3		5.7
" (4 " ").	41.5	41.3	38.5	27.8
" (4 " ") + 0.5 cc. 0.99 N H_2SO_4.	36.5	36.3	32.9	23.3
" (4 " ") + 1.5 " 0.99 N H_2SO_4.	28.6	28.2	25.5	14.4
" (5 " ").	40.4	40.7	36.0	26.2
" (5 " ") + 0.5 cc. N $NaHCO_3$.	45.2	44.8	42.1	32.0
" (5 " ") + 1.5 " N $NaHCO_3$.	54.2	53.9	52.0	41.7
Sheep (fresh).	50.7		46.4	
" + 0.5 cc. 1.2 N lactic acid.	45.2		40.1	
" + 1.0 " 1.2 N " "	37.6		33.8	
" + 1.5 " 1.2 N " "	31.8		28.1	

The indicated quantities of acid or alkali were added to 100 cc. portions
of blood, samples of which were then precipitated with picric acid. The
results show complete recovery of acid and alkali added.

Both high nitrogen and low cell volume may have had something
to do with the result.

That there should be a close parallelism between the variations
in the CO_2-combining power of the plasma, as determined, and the
change in titratable alkali of the blood was scarcely to be expected
in view of the differences in cell content and in percentage satura-

TABLE VI.

Effect of Intravenous Injections of Acid on the Titratable Alkali of the Arterial Blood and Plasma of the Dog.

	Plasma CO_2.		Titratable alkali. 0.1 N alkali per 100 cc. of blood or plasma.							
			Methyl red.		Neutral red.		Phenol red.		Thymol-phthalein.	
	Total.	BHCO₃	Blood.	Plasma.	Blood.	Plasma.	Blood.	Plasma.	Blood.	Plasma.
	per cent	*per cent*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*
Normal.............	49.0	46.7	42 0	37.2	38.3	35.8	36.2	34.5	26.6	30.4
N HCl	25.0	23.8	27.5	24.8	24.6	22.7	21.5	22.7	13.5	18.2
Normal.............	49.0	47.0	40.0	39 0	34 0	37.2	36.0	37.2	26.7	32.8
N lactic acid........	42.5	40.3	36.5	34.4	32.5	32.1	32.2	33.1	24.3	29.0
" " "	38.1	36.3	34 0	30.5	30 8	28.6	31.5	30.6	21.9	26.1
Normal.............	53.1	48.0	46.0				40.0			
0.52 N HCl..........	46.8	42.1	42.3				36.5			
0.52 " "	42.4	38.3	40.1				35.5			
0.52 " "	37.9	34.4	37.9				34.0			
0.52 " "	32.8	31.7	35.2				30.5			
0 52 " "	30.6	29.2	33.2				28.9			
Normal.............	45.4	44.2	39.9	37.2			35.6	36.0	27.0	29.9
0.55 N lactic acid......	39.3	37.8	35.6	33.0			29.3	31.0	22.2	23.9
0.55 " " "	33.3	31.7	31.7	30.3			26.4	28.3	17.7	20 2
0.55 " " "	23.9	21.9	25.5	23.8			19 0	20.7	12.2	13 2
0.55 " " "	14.6	12.9	16.8	18.4			10.6	14.7	2.25	5 6
0 55 " " "	11.4	10.1	13.4	16.1			8.2	12.7	−1.65	+1.7

The femoral artery of one leg and the femoral vein of the other were exposed under cocaine anesthesia. After taking samples of the arterial blood (under paraffin for examination of the plasma), the acid was slowly injected into the vein. 3 minutes after the injection had been interrupted, from 20 to 40 cc. of blood were run out of the artery to clear it and new samples were collected. The CO_2 determinations were made by the methods of Van Slyke and Cullen (1, 4, 5). In the first three experiments, the plasma was equilibrated with the operator's alveolar air for the determination of the combined CO_2, in the last this was calculated from the total CO_2 and the pH. This was, in the successive samples, 7.65, 7.50, 7.39, 7.15, 6.8 (approximately), 6.8 (approximately). The last two samples contained so much hemoglobin that comparison was difficult and probably inaccurate. The observed values were 6.8 and 6.8, but 7.0 has been used in the calculations. A correction of 0.18 has been used (13) to change the values at 20° to those at 38°.

tion with oxygen and carbon dioxide of the blood at the time the
plasma was separated. But that there is no closer parallelism
between any one of the three series of values for the titratable
alkali of the plasmas and the values for the CO_2-combining power
of the same plasmas is a matter that is not so readily explained.
Differences in protein content may account for some of these
deviations but there must be other factors, the nature of which is
not immediately apparent.

TABLE VII.

*Effect of Intravenous Injections of Sodium Carbonate upon Titratable Alkali
of the Arterial Blood of Dogs.*

	Plasma pH.	Plasma CO_2.		0.1 N alkali per 100 cc. of blood.		
		Total.	$BHCO_3$	Methyl red.	Phenol red.	Thymol-phthalein.
		per cent	*per cent*	*cc.*	*cc.*	*cc.*
Normal.....................	7.49	58.8	54.8	41.9	37.7	
Injected 5.4 per cent Na_2CO_3 .	7.61	97.0	90.6	57.0	51.3	
Normal....	7.39	65.6	61.1	39.2	33.9	28.4
Injected 5.4 per cent Na_2CO_3 .	7.58	104.3	97.5	59.1	55.6	45.9
Normal.....................	7.56	62.4	58.2	47.3	41.7	35.8
Injected 5.4 per cent Na_2CO_3 .	7.68	113.0	105.6	61.3	56.7	51.2

The experiments were conducted in the same manner as those with acid
(see note to Table VI). The pH was determined by Cullen's method, using
the correction 0.18 to change the values at 20° to those at 38°. The total
CO_2 was determined by Van Slyke's method and the combined CO_2 was
calculated. A report on the effect of the continuance of the injections of
the alkali will be found in the following paper.

In dog blood drawn from the artery and centrifuged under
paraffin and in sheep blood obtained at the slaughter house in
well filled bottles and centrifuged under paraffin, methyl red gave
higher values for whole blood than for plasma, phenol red gave
approximately the same for both, while thymolphthalein gave
lower values for whole blood than for plasma. A mixture of
phosphates at the reaction of the blood is alkaline to methyl red
and acid to thymolphthalein. The difference in reaction of the
end-points of these indicators is almost exactly that represented
by the second H^+ of phosphoric acid. The difference in the ti-

trations is about 10 cc. of 0.1 N NaOH per 100 cc. of blood and 7 cc. per 100 cc. of plasma.   If this were due entirely to phosphoric acid, it would require the presence of 31 mg. of inorganic phosphorus in the blood and 22 mg. in the plasma.   But there is only about one-fifth as much as this.   The organic "acid-soluble" phosphorus compound of the corpuscles may have sufficient buffer action to account for part of the buffer properties of the filtrate from blood but there is no considerable quantity of such a compound in plasma.

Experiments in which small quantities of amino-acids were added to picric acid and the mixture titrated to the end-points of the three indicators showed that these could account for a part of the buffer action.   Under conditions corresponding to a concentration of 33 mg. of amino-acid per 100 cc. of blood, the change in the buffer value of the picric acid solution was 0.30 cc. of 0.01 N NaOH with alanine and 0.48 cc. with glycine, equivalent to 1.0 and 1.6 cc., respectively, of 0.1 N alkali per 100 cc. of blood. Hiller and Van Slyke (14) found 8.3 mg. of amino nitrogen per 100 cc. of blood in the picric acid filtrates from ox blood.   This amount of nitrogen is the equivalent of 44 mg. of glycine or of 53 mg. of alanine.   Evidently, the amino nitrogen of the filtrate accounts for about 1.5 cc. of its buffer action.   Miller and Van Slyke also reported 4.6 mg. of peptide nitrogen per 100 cc. of blood in their picric acid filtrates.   Eckweiler, Noyes, and Falk (15) found a buffer value, between pH 6.0 and 9.0, of 42 cc. of 0.1 N NaOH per 100 cc. of a 0.05 N solution of dipeptide.   Such a solution contains 70 mg. of peptide nitrogen per 100 cc. or 15.2 times the concentration found in the blood.   Assuming that the molecular buffer action is no less in the dilute solution than in the concentrated, the peptide nitrogen of the picric acid filtrates will account for about 2.8 cc. of 0.1 N NaOH per 100 cc. of blood. The substances yielding peptide nitrogen in the analyses of Hiller and Van Slyke also yielded some amino nitrogen, but, after making allowances for such nitrogen, it is clear that the buffer action due to the amino-acids and peptides in the picric acid filtrates will amount to between 3 and 4 cc. of 0.1 N NaOH per 100 cc. of blood.

A similar examination of the figures for human blood shows similar relations to exist except that, in almost every instance,

TABLE VIII.

Comparison of Titratable Alkali of Blood and Plasma with CO_2-Combining Power of the Plasma.

Diagnosis.	Cell volume	Plasma CO_2.	Methyl red.		Phenol red.		Thymol-phthalein.	
			Blood.	Plasma.	Blood.	Plasma.	Blood.	Plasma.
Human blood.								
	per cent	per cent	cc.	cc.	cc.	cc.	cc.	cc.
Diabetes....................		15	18.2		13.1		3.6	
"	50.0	19	24.0		18.9			
Nephritis, N.P.N. 263..........	18.0	22	30.7	28.9	24.0	24.1	14.1	14.7
Diabetes....................	46.0	25	17.0		10.8		2.8	
"	50.0	32	27.2		23.7			
"	44.5	34	28.7		24.1			
Typhoid fever................	24.0	39	35.1	37.7	25.2	30.0		
Cardionephritis..............	26.3	39	38.1		31.8			
Diabetes....................	34.5	49	42.5	40.4	36.6	38.4	28.5	32.4
"	45.5	51	37.5		32.1		28.0	
Normal.....................	46.4	55	47.2		42.6		36.7	
"	48.0	56	43.9		39.2			
Tuberculosis of kidney.........	40.0	57	39.8	47.3	34.3	45.5	29.3	42.8
Normal.....................	50.0	60	39.1		33.4		25.9	
Diabetes....................	46.1	61	40.5		34.7		27.9	
Asthma.....................	46.9	61	44.3		37.4		29.2	
Normal.....................		62	44.3		38.6			
Uterine fibroids	36.7	62	47.0		40.8		33.7	
Exophthalmic goiter...........	48.0	62	41.9	44.1	36.5	40.7	29.4	36.6
Typhoid, pyelonephrosis........	49.4	63	49.5		44.2		40.3	
Chronic osteomyelitis, diabetes .	39.0	63	41.2	41.9	32.7	39.5	25.9	35.2
Normal.....................	50.0	63	42.1	44.6	33.5	41.6	31.0	37.9
"	40.7	63	42.7					
Pyelonephrosis (2 days after operation)................	42.0	64	42.3	44.9	36.2	42.6	29.4	40.2
Gastric neurosis..............	44.6	65	44.8		39.0		31.7	
Normal.....................	43.0	66	44.4	41.7	40.0	39.7	35.6	36.4
Diabetes....................	41.4	67	42.0	43.0	36.1	39.9	29.7	36.7
Auricular fibrillation..........	48.0	67	45.5	47.7	38.5	43.9	32.7	40.8
Unknown....................	40.0	67	46.8	49.0	39.0	44.2	31.3	39.4
Neurosis....................	49.0	67	43.9	44.7	38.2	42.2	30.8	37.4
1 week after hysterectomy and appendectomy..	34.8	69	45.4	51.6	38.9	47.8	33.4	45.2

TABLE VIII—*Concluded.*

Diagnosis.	Cell volume.	Plasma CO_2.	Methyl red.		Phenol red.		Thymol-phthalein.	
			Blood.	Plasma.	Blood.	Plasma.	Blood.	Plasma.

Human blood—*Concluded.*

Diagnosis.	Cell volume.	Plasma CO_2.	Methyl red Blood.	Methyl red Plasma.	Phenol red Blood.	Phenol red Plasma.	Thymol Blood.	Thymol Plasma.
	per cent	per cent	cc.	cc.	cc.	cc.	cc.	cc.
Chronic appendicitis	41.0	69	46.6	50.9	40.0	48.4	31.0	46.0
Diabetes	50.0	69	43.1	47.7	37.0	41.9	30.9	37.0
Cardiac insufficiency	45.0	70	43.9	47.3	39.3	45.5	32.5	42.8
Diabetes	30.0	70	46.6	46.9	39.5	44.3	34.8	42.0
"	44.0	73	44.8	47.7	40.1	45.0	34.0	40.7
Chronic osteomyelitis, diabetes	45.5	75	47.1		41.0		35.8	
Chronic bronchitis, emphysema	34.0	85	53.0	56.4	45.5	52.4	34.3	51.5

Sheep blood.

	Cell volume.	Plasma CO_2.	Methyl red Blood.	Methyl red Plasma.	Phenol red Blood.	Phenol red Plasma.	Thymol Blood.	Thymol Plasma.
1		63	49.6	48.1	43.0	43.4	40.3	42.0 (?)
2		59	48.1	46.3	44.2	42.0	39.0	36.8 (?)
3		63	49.1	48.9	44.8	44.4	35.0	38.2

TABLE IX.

Effect of the Addition of 1 Mg. of the Substances Indicated on the Titration of 5 Cc. of an Approximately 1 Per Cent Picric Acid Solution with 0.01 N NaOH.

	Methyl red.	Phenol red.	Thymol-phthalein.
	cc.	cc.	cc.
Picric acid	22.20	22.60	23.60
" " + 1 mg. alanine	22.30	22.75	24.00
" " + 1 " glycine	22.45	22.80	24.33
" " + 1 " creatine	22.40	22.80	23.85
" " + 1 " creatinine	22.10	22.80	23.90

the plasma gave higher values for titratable alkali with all three indicators. The animal blood was drawn from an artery, the human blood from a vein. As arterial blood becomes venous in character, Cl (and possibly other anions) pass from the plasma into the cells and it is, therefore, not surprising that plasma obtained from venous blood should contain more titratable alkali

Titratable Alkali of the Blood

TABLE X.

Comparison of Determinations of Titratable Alkali by Gravimetric Method, Using Nitron, and by Volumetric Method, Using Titanous Chloride (Methyl Red Only).

Gravimetric method. 5 cc. blood precipitated 30 cc. filtrate, = 3 cc. blood, used. 0.1 N alkali per 100 cc. of blood	Volumetric method.		Value obtained. 0.1 N alkali per 100 cc. of blood.		
	Amount of blood pre-cipitated.	Equivalent in liquid analyzed.	I	II	Average.
cc.	cc.	cc.	cc.	cc.	cc.
47.7	0.30	0.200	45.6	48.9	47.3
	0.40	0.225	48.5	48.0	48.3
	0.50	0.300	46.9	45.5	46.2
46.0	0.30	0.175	46.2	44.7	45.5
	0.40	0.225	45.3	46.2	45.8
	0.50	0.318	46.6	44.9	45.8
46.2	0.30	0.200	43.8	45.3	44.6
	0.40	0.250	43.9	44.9	44.4
	0.50	0.338	45.7	44.6	45.7
46.0	0.30	0.175	44.7	44.7	44.7
	0.40	0.250	44.0	44.8	44.4
	0.50	0.325	45.8	44.3	45.1
47.5	0.30	0.175	47.7	46.0	46.9
	0.40	0.235	46.0	45.8	45.9
	0.50	0.300	49.8	47.4	48.6
47.3	0.30	0.175	42.0	44.8	43.4
	0.40	0.225	46.6	45.8	46.2
	0.50	0.300	48.1	46.7	47.4

TABLE XI.

Comparison of Results Obtained with Blood from an Arm Vein and with Quantities of 0.2 to 0.5 Cc. from Fingers, Showing Comparative In-accuracy of the Method, Apparently Due to Tissue Material Introduced by Squeezing Finger (Methyl Red Only).

Vein.	Fingers.				
	I	II	III	IV	V
42.7	50.7	49.3	45.3	47.1 ·	47.2
37.1	41.5	44.1	37.7	34.9	38.4
47.2	48.3	48.9	52.7	48.7	50.2

than that obtained from arterial blood. The figures in Tables VI and VIII indicate a transfer of the equivalent of 2 or 3 cc. of 0.1 N acid from plasma to corpuscles as the blood passes from the arteries to the veins. This is about the amount that is indicated by direct experiment (16).

EXPERIMENTAL.

Preparation of Reagents.

Picric Acid.—Merck's "blue label" picric acid is satisfactory. Dissolve 20 gm. in hot water in a 2,000 cc. volumetric flask, allow to cool, and dilute to mark.

0.01 and 0.005 N NaOH.—Prepared daily by dilution of 0.1 N NaOH with CO_2-free H_2O.

Indicators.—*Methyl red* and *phenol red.* 0.4 per cent solutions are prepared according to Clark (17) by dissolving 0.1 gm. in 7.4 and 5.7 cc., respectively, of 0.05 N NaOH and diluting to 25 cc. For use in this method, these solutions are diluted with 9 volumes of water.

Thymolphthalein.—A 1 per cent solution in alcohol, to which there is added enough NaOH to give a slight blue color.

Nitron.—A filtered 1 per cent solution in 10 per cent acetic acid. *Recovery of excess nitron from the filtrates.* These are evaporated to small volume, cooled, and made strongly alkaline with ammonia. The nitron separates out, is filtered, washed with a little water, air-dried, and is then ready for use.

Titanous Chloride.[3]—To 70 cc. of commercial 20 per cent $TiCl_3$, add 140 cc. of concentrated HCl, heat to boiling for 1 minute, cool, and dilute to 2,000 cc. with water that has been boiled and cooled under carbon dioxide.

Ferric Ammonium Sulfate.[3]—Mix 50 cc. of concentrated H_2SO_4 with 2,000 cc. of H_2O, heat to boiling, cool under CO_2, and use to dissolve 48.4 gm. of $Fe_2 (SO_4)_3. (NH_4)_2 SO_4.24 H_2O$.

NH_4CNS.[3]—Dissolve 100 gm. in air-free water and dilute to 1,000 cc.

The $TiCl_3$, $Fe_2 (SO_4)_3$, and NH_4CNS are kept in bottles to which there are attached burettes and hydrogen generators as shown in Fig. 1.

SUMMARY.

A method based upon a new principle, for the determination of the titratable alkali of the blood is described. The results are unaffected by the presence of small amounts of oxalate or by the degree of saturation of the blood with oxygen or with carbon diox-

---

[3] For use with the volumetric method only.

ide. Several indicators may be used in the same sample. With
phenol red, the end-point is approximately at the reaction of
human blood and the value then obtained represents the amount

Fig. 1.

of alkali previously combined with the carbonic acid and protein
of the blood. For normal human blood, this is the equivalent of
approximately 38 cc. of 0.1 x NaOH per 100 ec. of blood. By a
suitable choice of indicators, the value of the "acid-soluble"

buffers may be determined.   This is about 11 cc. of 0.1 N NaOH per 100 cc. of blood between the end-points for methyl red (pH 6.0) and thymolphthalein (pH 9.0).   The error in the method is ± 1 per cent with 5 cc. of blood, ± 2 per cent with 1.6 cc. of blood, and ± 4 per cent with from 0.3 to 0.5 cc. of blood.   These figures apply to the titration with methyl red.   They are slightly greater to other indicators and with the very small quantities of blood only methyl red was found suitable.

BIBLIOGRAPHY.

1. Van Slyke, D. D., and Cullen, G. E., *J. Biol. Chem.*, 1917, xxx, 289.
2. Joffe, J., and Poulton, E. P., *J. Physiol.*, 1920–21, liv, 129.
3. Peters, J. P., Jr., and Barr, D. P., *J. Biol. Chem.*, 1920–21, xlv, 537.
4. Van Slyke, D. D., *J. Biol. Chem.*, 1922, lii, 495.
5. Cullen, G. E., *J. Biol. Chem.*, 1922, lii, 501.
6. Henderson, Y., and Morriss, W. H., *J. Biol. Chem.*, 1917, xxxi, 217.
7. Busch, M., and Blume, G., *Z. angew. Chem.*, 1908, xxi, 354.
8. Knecht, E., and Hibbert, E., *Ber. chem. Ges.*, 1907, xl, 3819.
9. English, F. L., *J. Ind. and Eng. Chem.*, 1920, xii, 994.
10. Kay, P., and Appleyard, J., *J. Soc. Dyers and Colourists*, 1888, iv, 83.
11. Peters, J. P., Jr., Barr, D. P., and Rule, F. D., *J. Biol. Chem.*, 1920–21, xlv, 489.
12. Evans, C. L., *J. Physiol.*, 1922, lvi, 146.
13. Cullen, G. E., *J. Biol. Chem.*, 1922, l, p. xvii.
14. Hiller, A., and Van Slyke, D. D., *J. Biol. Chem.*, 1922, liii, 253.
15. Eckweiler, H., Noyes, H. M., and Falk, K. G., *J. Gen. Physiol.*, 1920–21, iii, 291.
16. Doisy, E. A., and Eaton, E. P., *J. Biol. Chem.*, 1921, xlvii, 377; 1922, l, p. xv.
17. Clark, W. M., The determination of hydrogen ions, Baltimore, 1920.

THE SUPPOSED RELATION BETWEEN ALKALOSIS AND TETANY.*

BY ISIDOR GREENWALD.

(*From the Harriman Research Laboratory, Roosevelt Hospital, New York.*)

INTRODUCTION.

In the past few years, there have been a number of observations reported from which it has been concluded that a too alkaline condition of the blood and, consequently, of other tissue fluids resulted in electrical hyperexcitability, tetany, etc. This too alkaline condition could be a result of parathyroidectomy. intravenous administration of sodium bicarbonate, forced breathing, persistent vomiting, gastric fistula, etc. This conception has probably found its most explicit expression in the discussion of "the normal and abnormal variations in the acid-base balance of the blood" by Van Slyke (1) in which it is definitely stated that a hydrogen ion concentration of pH 7.9, whether caused by an uncompensated CO_2 deficit or by an uncompensated alkali excess, induces tetany.

Tetany after Parathyroidectomy.

The idea that "alkalosis" and tetany are related seems to have had its origin in the work of Wilson and his collaborators (2, 3, 4), who found that injection of hydrochloric acid relieved the tetany of parathyroidectomized dogs, and that after parathyroidectomy "there is usually a sudden diminution in the excretion of acids and ammonia and a decrease in the ammonia ratio and the hydrogen ion concentration of the urine," and an increase in the value of K in Barcroft's equation for the relation between

---

* A preliminary report appeared in the Proceedings of the Society for Experimental Biology and Medicine (Greenwald, I., *Proc. Soc. Exp. Biol. and Med.*, 1920–21, xviii, 228).

the percentage saturation of the blood with oxygen and the tension of oxygen with which it is in equilibrium, which increase they interpreted as an indication of an increased alkalinity of the blood.

That injections of acid relieve tetany is not of special significance. So do injections of calcium, magnesium (5), strontium, and barium (6) salts, or potassium or sodium chloride (7). Moreover, such relief is frequently spontaneous.

The changes in the urine reported by Wilson, Stearns, and Janney (4) are sufficiently readily explained by the observations published in 1911 and 1913 by the present writer (8, 9). It was then found that, immediately after parathyroidectomy in dogs, there was a very marked retention of phosphorus, the amount in the urine falling to as little as 2 per cent of its former value, and that this retention was primary and was followed, only after 1 or 2 days, by a retention of sodium and potassium. Obviously, the acidity of the urine should diminish unless the failure of excretion of the phosphoric acid was compensated for by the sudden appearance of large quantities of some other acid. And this diminished acidity should not have been regarded as a sign of "*alkalosis*" but as evidence of *acidosis*. The retention of phosphoric acid was demonstrated by the present writer (10) to be accompanied by an increased concentration of phosphoric acid, or similar compound, in the blood. A considerable amount of alkali would be required to neutralize this. An increased formation of guanidines has since been reported by Paton and his co-workers (11), but they have not claimed to have found anything like the quantity required to neutralize the phosphoric acid retained, to say nothing of securing an increased alkalinity.

The changes in the blood reported by Wilson, Stearns, and Thurlow (3) merit fuller consideration. Since they worked at zero CO_2 tension, their results on the oxygen saturation of the blood could not demonstrate a change in the reaction of the circulating blood but only in what has since been called "alkaline reserve" or, better, CO_2-combining power. There may or may not have been an actual change in reaction. Their own results with a dialysis indicator method (12) showed that there was no demonstrable change in the reaction. There was a change in the reaction of the dialysate obtained after shaking out "the excess of carbon dioxide" which might indicate an increased CO_2-combining power.

From his work on human blood, Barcroft (13) derived the formula $\frac{y}{100} = \frac{K\,x^n}{1-K\,x^n}$ as an expression for the relation between the pressure of oxygen, in millimeters of mercury (x), with which the blood was in equilibrium and its percentage saturation with oxygen (y). Barcroft found that (n) had the value 2.5. (This has recently been changed by Hill (14) to 2.2.) The value of (K) varied with the nature of the blood and was particularly markedly affected by the reaction, becoming greater as the alkalinity was increased.

In their calculations, Wilson, Stearns, and Thurlow used the same value for (n) for dog blood as Barcroft had for human blood. But calculations from their own results show that, under the conditions they employed, the value of (n) was not 2.5, but approximately 1.5. Only then does the value of (K) for the normal dog blood approach constancy $(17 \times 10^{-3}$ to $27 \times 10^{-3})$. But if this value for (n) be used in calculations from the data obtained with parathyroidectomized dogs, it is found that only rarely is the value of (K) thus obtained much greater than the normal for that animal or outside the normal range.

The significance of the few changes in the value of (K) as remain when the correct value of (n) is used is obscure. Barcroft found the value of (n) for hemoglobin in NaCl solution to be 1.778, in KCl solution, 2.49. Even a slight decrease in the value of (n) would greatly increase the apparent value of (K) and it seems quite possible that the change in the concentration of calcium or of phosphorus in the blood should have been responsible for the apparent increase in the value of (K).

The effect on the reaction of the dialysate of the blood shaken to remove "excess of carbon dioxide" must have been due to other causes than an increased CO_2-combining power because such change could hardly fail of detection by its effect on the value of (K); unless, indeed, it were masked by a simultaneous increase in the value of (n). It is possible that the change in the calcium and phosphorus of the blood, by changing the error in the dialysis indicator method due to the Donnan equilibrium, should have been responsible for the apparently increased CO_2-combining power.

There would seem to be, therefore, nothing in the work of Wilson and his associates that is really indicative of a relation between alkalosis and tetany. But the hypothesis seems to have been an attractive one and even the work of Hastings and Murray (15) and of Underhill and Nellans (16), which has conclusively shown that there is, after parathyroidectomy, no change either in alkalinity or in CO_2-combining power, has not caused it to be abandoned.

Tetany after Administration of Alkalies.

It is, of course, possible that other types of tetany, such as those occurring after persistent vomiting, hyperpnea, or injection of alkalies, are due to alkalosis, even if the tetany of parathyroidectomized dogs is not. However, no such effect following the administration of alkalies is mentioned by Hougardy (17) or by Scott (18). The former used sodium hydroxide and observed the occurrence of apnea, which was more marked, or obtained with smaller doses, if the injection was made into the carotid artery towards the head than if it was made into a vein and was least pronounced, or required the largest dose, when the injection was made through a sound inserted through the carotid artery into the aorta. Sodium carbonate and calcium hydroxide produced similar effects but in none of his protocols did Hougardy mention the appearance of tetany.

Scott injected sodium carbonate into decerebrate cats and obtained considerable increases in CO_2-combining power and in alkalinity of the blood, as measured by a dialysis indicator method. However, in the course of the work to be presented in this paper, not quite such great changes were obtained by a more accurate method, even after the injection of much larger quantities of sodium carbonate. Therefore, it seems quite likely that the magnitude of the changes observed by Scott was due, in part, to a greater escape of carbon dioxide from the specimens which were under a high CO_2 tension than from those collected under normal conditions. Even if Scott's data are absolutely correct, they only show that the reaction of the blood of decerebrate cats may be changed from a normal of pH 7.3 to 7.4 to pH 7.7 to 7.8 without the appearance of tetany, for it is scarcely conceivable that such an occurrence could have escaped his observation and description.

However, it may be objected that the removal of the cerebrum prevented the appearance of tetany.

Although experimental evidence was lacking, it was assumed by some that the tetany that sometimes appeared after the therapeutic administration of sodium bicarbonate was due to alkalosis. Palmer and Van Slyke (19) advised great caution in the use of sodium bicarbonate and regarded a plasma CO_2-combining power of 80 per cent as dangerous. At the most, this could mean only a slight change in actual hydrogen ion concentration. Assuming that the alveolar CO_2 tension remained at 40 mm., there would be 2.84 per cent free carbon dioxide in the plasma. From Hasselbalch's formula, this would mean a pH of 7.55, if the CO_2-combining power were 80 per cent; if the pH is assumed to be constant at 7.4, the 80 volumes per cent of bound carbon dioxide would require a free carbon dioxide content of 5.9 per cent, equivalent to an alveolar CO_2 tension of 83 mm. Neither of these adjustments should be so readily assumed to be outside the capacity of the organism, particularly since both are probably operative and, consequently, neither need be fully exercised.

It is interesting to observe that the cases of tetany that have been reported to have occurred after the therapeutic administration of sodium bicarbonate (20 to 23) were all in individuals in whom renal deficiency was known to exist or was quite probable. If the kidneys cannot excrete the excess alkali, it is retained. But there is little or no increase in alkalinity for there is a retention of carbon dioxide that keeps the reaction normal, or nearly so. *In no case of tetany following the administration of sodium bicarbonate has a change in the reaction of the blood been demonstrated.*

In order to obtain some definite knowledge regarding the possibility of producing either a change in the reaction of the blood or tetany, or both, by the injection of sodium bicarbonate or carbonate, a number of experiments were performed upon dogs. The results embodied in a preliminary report made in 1921, showed that huge amounts of sodium bicarbonate were required to produce tetany and that there was, at the most, only a slight change in the reaction of the blood. Unfortunately, zinc sulfate was used as the anticoagulant and this made all the values for the pH too low. The same amount of zinc sulfate was used in each case and should, theoretically, have produced a greater

change in pH in normal blood than in that obtained after administration of sodium bicarbonate. However, there remained some doubt as to the significance of the results.

The experiments were repeated several times with improved technique and with the same results. Too rapid injection of either sodium carbonate or bicarbonate was followed by apnea, which might be fatal. If this were avoided, there was no marked effect except an increased heart rate until very large amounts had been injected and until the bicarbonate content of the plasma had reached a very high level, over 120 volumes per cent. The reaction of the plasma of the arterial blood was determined by Cullen's method (24) and was found to have changed only slightly, not more than 0.23 on the pH scale. As the injection was continued, twitching and tremor and, finally tonic-clonic convulsions appeared. Blood taken from the artery as soon as possible after the appearance of the convulsions showed that there had been no further change in the reaction of the blood. As the convulsions continued, the reaction of the blood became less and less alkaline, the concentration of total carbon dioxide in the plasma fell rapidly, until samples of blood taken shortly before the animal died showed a condition of *acidosis*, and not alkalosis, to obtain. In one experiment (Table III), the pH of the plasma changed from 7.56 before the injection of sodium carbonate was begun to 7.69 just before the convulsions appeared, and then rapidly fell to 7.04 just before death. The corresponding figures for the carbon dioxide *content* were 62.4, 171, and 54.1 volumes per cent and for the *bicarbonate* carbon dioxide were 60.4, 167, and 48.2 volumes per cent. The results obtained in the determination of titratable alkali by the method of Greenwald and Lewman (25), which measures essentially the amount of base combined with $'HCO_3$ and with protein in the blood, were very similar, the titrations to phenol red changing from 41.7 to 77.3, and then to 28.6 cc. of 0.1 n NaOH per 100 cc. of blood.

The relative constancy of the reaction of the plasma, in spite of the great increase in bicarbonate content, could be secured only by a retention of free carbon dioxide. No determinations of the alveolar carbon dioxide tension were made but calculations from the total carbon dioxide and pH of the plasma indicate that, when the bicarbonate content of the plasma was at its highest,

the alveolar carbon dioxide tension was about 60 mm. of mercury. After the animal went into convulsions, large amounts of carbonic, lactic, and, probably, other acids were formed and the calculated carbon dioxide tension rose to as much as 100 mm.

TABLE I.

Dog 8.   Female mongrel.   Weight 15 kilos.   Used 5.01 per cent $NaHCO_3$.

Time. Nov. 18, 1921.	Injected.	Total.	Heart rate.	Respiratory rate.	Blood. Titratable alkali. Methyl red.	Phenol red.	Cell volume.	Total plasma CO_2.	Remarks.
a.m.	cc.	cc.					per cent	per cent	
10.00					43.7	36.8	47.9	52.4	
10.02			120	15					Washed bladder.
10.07–10.11	75	75							
10.14					51.5	44.3	45.1	77.2	
10.15–10.19	75	150							
10.21			120	20					
10.24					57.7	51.5	44.6	95.2	
10.25–10.33	150	300							
10.34			240	20					
10.36					70.5	64.0	40.0	123	
10.37–10.45	150	450							
10.48			270	21	76.9	69.9	40.9	136	
10.51–11.05	225	675			.				
11.08			225	32	83.5	78.8	38.2	154	
11.09–11.18	150	825							
11.15–11.24									Twitching, increasing to convulsions.
11.25					68.0	61.0	37.2	132	Convulsions.
11.30					57.9	48.0	37.6	104	"
11.35					51.5	38.8		77.2	"
11.44					43.0	27.5	39.1	61.6	"
11.46								.	Respiration ceased.

Urine.   Volume 485 cc. titrated as 0.1865 N alkali, after removing CO_2. Contained 2.16 gm. of sodium, 0.349 gm. of potassium, 0.537 gm. of chlorine, and 0.0112 gm. of phosphorus.

Injected.............11.30 gm. of sodium or 0.754 gm. per kilo.
Excreted............. 2.16 „" " "
Retained in body ..... 9.14 " " " or 0.609 gm. per kilo.

TABLE II.

Dog 11.   Male fox-terrier.   Weight 10.56 kilos.   Used 5.36 per cent Na_2CO_3.

Time. Mar. 16, 1922.	Injected.	Total.	Heart rate.	Respiratory rate.	Blood. Titratable alkali. Methyl red.	Phenol red.	Thymolphthalein.	Lactic acid per 100 cc. mg.	cc. 0.1 N	Plasma. pH	Total. per cent	BHCO₃ per cent	Free. per cent	Tension. mm.	Sodium per 100 cc. mg.	Remarks.
a.m.																
10.04	70	70			41.9	37.7				7.49	58.8	56.5	2.3	32		Washed bladder.
10.14			200	40												
10.15–10.42	70	140														
10.45			210	30	57.0	51.3		45.7	5.08	7.61	97.0	94.1	2.9	41		
10.48																
10.49–10.56																
10.58	140	280														
10.59			250	20	67.4	63.4		32.6	3.62	7.72	148.0	144.5	3.5	49		
11.00–11.18																
11.20					83.1	78.5		73.4	8.16							
11.21			180	32												
11.23																
11.24–11.30	38	318														
11.33			150	30	86.3	82.9		88.2	9.80	7.72	190.0	185.6	4.4	62		

													Tremor.
11.34–11.41	52	370											
11.42	8	378											
11.43			85.0	83.5	70.6	201	22.3	7.66	196.0	190.5	5.5	77	512	Convulsions.
11.45			71.0	66.5	51.6	297	33.0	7.64	150.0	145.8	4.2	59	500	"
11.49			58.9	53.7	28.8	376	41.8	7.39	111.5	106.0	5.5	77	502	"
11.56														"
p.m.														Inspiratory spasm. Arti-
12.01			50.6	41.4	18.1	385	42.8	7.38	82.0	77.9	4.1	58	552	ficial respiration not suc-
12.07														cessful. Heart slowed and stopped.

Urine.   Volume 305 cc.

Titrated, after removal of CO_2 as 0.21 N alkali.

Contained 0.228 gm. of chlorine. 0.067 gm. of lactic acid, 0.018 gm. of phosphorus, 0.263 gm. of potassium, and 1.35 gm. of sodium (1.50 gm. by Kramer's method).

Injected..8.78 gm. of sodium or 0.832 gm. per kilo.

Excreted..1.42 " " " "

Retained in body..................................7.36 " " or 0.698 gm. per kilo.

TABLE III.

Dog 13. Male beagle. Weight 17.0 kilos. Used 5.36 per cent Na$_2$CO$_3$.

Time, Mar. 31, 1922. (a.m.)	Injected.	Total.	Heart rate.	Respiratory rate.	Blood — Titratable alkali, Methyl red.	Phenol red.	Thymolphthalein.	Lactic acid per 100 cc. (cc 0.1 N)	Lactic acid per 100 cc. (mg.)	Cell volume. (per cent)	pH	Plasma — Carbon dioxide, Total. (per cent)	BHCO$_3$. (per cent)	Free. (per cent)	Tension. (mm.)	Sodium per 100 cc. (mg.)	Remarks.
10.11					47.3	41.7	35.8	2.78	25.0	46.3	7.56	62.4	60.4	2.0	28	305	
10.20			125														
10.21				18													
10.23–10.52	136	136															
10.55			115														
10.58				16													Washed bladder.
11.00–11.22	204	340			61.3	56.7	51.2	2.71	24.3	37.0	7.68	113.0	110.0	3.0	42	388	
11.23			190														
11.24				13													
11.26–11.45	136	476			74.3	70.9	61.6	7.68	69.1	37.8	7.87	159.2	155.4	3.8	53	404	
11.48			180	20	80.0	77.3	68.9	7.63	68.7	39.5	7.69	171.0	166.8	4.2	59	475	
11.51 a.m.–12.13 p.m.	187	663															Faint tremor; changed receiver for urine. Rectal temperature 39.5°. Twitching gradually increasing to tonic-clonic convulsions at 12.15 p.m.

p.m.														
12.02–12.15														Changed receiver for urine at 12.15 p.m.
12.16	71.8	66.3	57.3	244	27.1	10.1	7.45	162.2	155.3	6.9	97	547		Convulsions.
12.20	51.0	47.0	40.0	300	33.3	11.3	7.41	108.8	103.7	5.1	72	527		" " Rectal temperature 39.8°
12.27	53.4	45.0	31.3	376	41.8	45.6	7.29	91.5	88.8	5.7	81	548		" " " 40.0°
12.30	43.2	35.9	24.7	417	46.3		7.14	85.8	78.6	7.2	101			" "
12.35	41.9	33.5	17.6	401	44.6	8.1	7.16	63.3	58.2	5.1	72			" "
12.44	34.9	28.6	12.6	461	51.2	46.2	7.01	51.1	48.4	5.9	80			Rectal temperature 40.5°

Urine.	Volume.	Alkalinity.		Chlorine.	Phosphorus.	Lactic acid.	Potassium.	Sodium.	
		pH	Titration.					Author's method.	Kramer's method.
	cc.		N	gm.	gm.	gm.	gm.	gm.	gm
I	690	8.55	0.225	0.760	0.005	0.025	0.469	3.52	3.75
II	251	8.55	0.256	0.251	0.026	0.502	0.122	1.39	1.35
III	12	8.57				0.043			0.024

Injected to first sign of tremor 11.10 gm. of sodium or 0.653 gm. per kilo; to convulsions 15.40 gm. or 0.940 gm. per kilo.

Excreted................. 3.64 " " " " 5.01

Retained in body.......... 7.46 " " " " or 0.439 gm. per kilo; to convulsions 10.36 gm. or 0.610 gm. per kilo.

It is of interest to notice that the concentration of lactic acid in the blood was increased before the tetany was observed.   That the administration of alkalies increases the amount of lactic acid in the urine has been reported by Macleod and Knapp (26).

Examination of the values obtained in the determination of what Greenwald and Lewman (25) have called titratable alkali, using different indicators, discloses some interesting relations.   In Experiment 13 (Table III), the normal values were 47.3, 41.7, and 35.8 cc. of 0.1 N alkali per 100 cc. of blood, to methyl red, phenol red, and thymolphthalein, respectively.   Just before death, they were 34.9, 28.6, and 12.6.   The difference between the titration to methyl red (pH 6.0) and that to phenol red (pH 7.4) was little changed but that between the latter and the titration to thymolphthalein (pH 9.0) was greatly increased.   This could not have been due to the lactic acid because this is quantitatively titrated with methyl red.   Since the disturbance is entirely on the alkaline side of the phenol red end-point, it must have been due to the presence of weak bases or to acids having a dissociation constant lower than that of the second hydrogen ion of phosphoric acid, which gives a symmetrical curve.   Since the excretion into the urine during the convulsions is negligible the presence of some other acid is also indicated by the fact that the increase in the concentration of lactic acid in the blood is not sufficient to account for the observed fall in the titratable alkali.   Both blood and urine were examined for β-hydroxybutyric acid and the latter for acetoacetic acid, also.   Neither was found in amounts at all comparable with those required to account for that part of the fall in blood alkali which was not due to the increased concentration of lactic acid.   The nature of the substance remains unknown.

The tetany and convulsions following the injection of sodium carbonate are evidently not due to alkalosis.   To what may they be ascribed?   Examination of Table IV leaves little doubt.   When convulsions appear after the injection of sodium carbonate or bicarbonate, the concentration of sodium in the plasma is the same as when convulsions appear after the injection of sodium chloride or sulfate.   All sodium salts injected in large excess are toxic and, apparently, several of them are about equally toxic.   In each case, there is produced a sudden and marked disturbance of the relation between sodium ion and other cations.   Osmotic

TABLE IV.

Comparison of Toxicity of Several Sodium Salts.

Experiment No.	Salt.	Concentration of sodium per liter.	Time.	Sodium. Injected per kilo.	Retained per kilo.	Per 100 cc. plasma.	Remarks.
		gm.	*min.*	*gm.*	*gm.*	*gm.*	
V*	NaCl	11.5	26	0.995	0.832	0.408	Twitching.
		13.7	33	1.455	1.302	0.493	Convulsions.
VI*	Na₂HPO₄ / NaH₂PO₄	8.73	31	0.702	0.621	0.451	Respiratory failure, no twitching.
VII*	Na₂SO₄	5.84	106	1.285	0.822	0.480	Twitching.
1	NaHCO₃	21.8	38	0.694	0.493	0.442	"
3	NaHCO₃	21.8	40	0.793	0.709	0.457	Convulsions, in spite of use of 45 mg. K, 32 mg. Ca, and 6 mg. Mg per kilo injected into another vein.
4	NaHCO₃	10.9	68	1.140	0.870	0.420	Respiratory failure, no twitching.
5	NaHCO₃	21.5	31	0.747	0.730	0.436	Twitching.
	NaCl in HCl		41	0.807	0.790	0.452	Convulsions not checked by 2.59 cc. N HCl in N NaCl per kilo.
7	NaHCO₃	21.5	51	0.761	0.591	0.455	Twitching.
			66	1.100	0.866	0.533	Convulsions, which were not checked by subsequent injection of 1.30 cc. N HCl in N
	NaCl in HCl		84	1.130	0.889	0.496	NaCl per kilo.
8	NaHCO₃	13.7	105	0.754	0.609		Convulsions.
9	NaHCO₃	15.5	77	0.932	0.706	0.476	"
11	Na₂CO₃	23.2	90	0.832	0.698	0.512	"
13	Na₂CO₃	23.2	87	0.653	0.439	0.475	Faint tremor.
			114	0.940	0.609	0.547	Convulsions.

* See Greenwald (27).

pressure plays a part and the nature of the anion is not without significance. It must be remembered that it is not the concentration of sodium in the plasma that is, in itself, significant but that it is the concentration in certain cells or at their boundaries that determines the effects obtained. The toxic action may depend upon the ability of the sodium ion to penetrate the cell and thus upset the ionic equilibrium therein or it may depend upon its being unable to penetrate, while combined with an anion that does and in that manner damaging the cell by virtue of the electrical disturbance thus produced at the surface. But whatever the precise mechanism may be, there seems to be little doubt but that the convulsions following the injection of sodium carbonate are due to what the author (27) has called "sodium poisoning," a disturbance, due to excess of sodium ion, of the normal relations between this and other cations.

Gastric Tetany.

There remain to be considered two types of tetany, both of which appear to be due to a loss of acid from the body. These are gastric tetany and the tetany of hyperpnea. The former of these occurs after persistent vomiting or excessive gastric lavage which involve the loss of considerable amounts of hydrochloric acid. It is not surprising to find an increase in the CO_2-combining power of the plasma. *But there is no, or very little, change in the reaction* (28). What happens is merely an exaggeration of what happens after every meal. There is a secretion of hydrochloric acid into the stomach and the loss of this acid from the blood is compensated for, in part, by the excretion of a more alkaline urine, and in part, by a retention of carbon dioxide. The process cannot be continued indefinitely unless the hydrochloric acid is later reabsorbed. If it is not, tetany and collapse occur sooner or later. But these are not due to alkalosis but to the marked disturbance in ionic equilibrium brought about by the loss of so much chlorine. That ammonium chloride should relieve gastric tetany is not surprising (29). It supplies the missing chlor-ion, combined with a base that is readily converted into a neutral substance, and thus restores the ionic equilibrium.

Tetany and convulsions are not due to any single cause. Any one of a multitude of disturbances in the equilibrium within

certain tissues may be responsible. Convulsions are to be regarded as a sign of approaching or partial disintegration of the neuromuscular apparatus. The defect may occur in any one of several structures and may be due to any one of many causes.

Tetany Following Hyperpnea.

Collip and Backus (30) observed tetany in a few of fifteen individuals who subjected themselves to voluntary forced breathing. The CO_2-combining power of the plasma and the CO_2 tension of the alveolar air were determined before, immediately after, and at intervals after, the period of hyperpnea. Neither the reaction nor the total carbon dioxide of the plasma were determined but the ratio of free to combined carbon dioxide in the plasma was calculated upon the assumption that the alveolar air was in equilibrium with the plasma. The author has calculated from this ratio, assuming the value of 6.1 for pK_1 in Hasselbalch's equation, the pH of the plasma, which appears to have been changed, by the forced respiration, by from 0.13 to 0.39, the average being 0.24. The maximum value of pH calculated is 7.82. The actual change may have been even less for, after a period of hyperpnea, the usual period of contact may not be sufficient to bring the alveolar air into equilibrium with the blood. However, there must have been some change for the character of the urine changed, becoming more alkaline in spite of the diminished excretion of ammonia and increased excretion of phosphates.

Grant and Goldman (31) performed a smaller series of similar experiments. They determined the pH by a dialysis indicator method and found, in five experiments, a change of from 0.05 to 0.25, the average being 0.16. The highest value of pH observed was 7.65.

Collip and Backus and Grant and Goldman accepted this change in reaction as an explanation of the tetany observed. In a subsequent publication, in which he dealt with the effects of the subarachnoid and intraarterial injection of sodium bicarbonate and other electrolytes, Collip (32) emphasized the disturbance in the cation equilibrium, although he also ascribed a specific stimulating effect of the bicarbonate anion.

It is interesting to contrast the attitude of British investigators with that of the Americans.

Davies, Haldane, and Kennaway (33) observed tetany-like symptoms after forced breathing and they recognized that there was probably a slight change in the reaction of the blood.   However, they recognized that this was not, in itself, the cause of the tetany.   They stated:

"There would however have been comparatively little alkalosis in the tissues, as Yandell Henderson has shown in numerous papers on acapnia and shock that forced breathing decreases the peripheral circulation. The bad effects noticed by us were relieved by inhalation of oxygen and were presumably due partly to increased stability of oxyhæmoglobin, partly to vaso-constriction."

Hyperpnea induced by hot baths was found by Bazett and Haldane (34) to produce tetany-like symptoms,

"which were relieved by breathing mixtures containing either 8.5 p. c. CO_2 with 14.2 p. c. O_2, or equal volumes of air and O_2.   They were therefore probably due to oxygen want in the tissues caused by over-stability of oxyhæmoglobin resulting from acapnia, as in voluntary forced breathing."

This view is made more probable by the work of Morris (35), who found that a number of manipulations, which had in common, apparently, only the fact that they all produced anoxemia in the tissues, all increased the electrical excitability of the neuromyon.

In fact, all these observations were clearly foreshadowed by the work of Hill and Flack (36), who wrote in 1910:

"The inhalation of oxygen lessens the discomfort of forced breathing. It enables young men with a great power of pulmonary ventilation to go on with forced breathing for as long as 19 minutes, and to wash the CO_2 out of the body till the alveolar tension sinks to as low as 1.47%."

The lowest alveolar CO_2 content observed by Collip and Backus was 2.1 per cent and the average, after hyperpnea, was 2.9.   The corresponding figures in the experiments of Grant and Goldman were 2.47 and 2.76, respectively.   But Hill and Flack were able to lower it to 1.47 per cent without the appearance of tetany, because there was no anoxemia.

Paradoxical as it may seem, there can be little doubt but that forced breathing produces tissue asphyxia.   It is this and not the increased alkalinity, *per se*, that induces tetany.

It would appear that as long as life exists at all, the reaction of the blood can be changed only to a very slight degree. Such changes as do occur seem to affect, directly, only the respiration. It is the respiratory center that is, next to the buffer properties of the blood itself, the immediately active regulator of the reaction of the blood. The kidneys act more slowly. The respiratory center can be set aside, as in forced respiration, or given too great a task, as in intravenous administration of acid or of alkali, but, ordinarily, it functions quickly and adequately and maintains the reaction of the blood at a nearly constant value.

EXPERIMENTAL.

After a fast of 1 or 2 days, the dog was catheterized and, under cocaine anesthesia, the femoral artery of one side and the vein of the other were exposed and attached to cannulæ. A sample of blood was taken from the artery. The bladder was washed with distilled water and the injection of the solution was then begun. In the earlier experiments (Experiments 1 to 7), the samples of blood were large, about 200 cc., and no others were drawn until marked symptoms had developed. In the later experiments however, the samples were smaller, not exceeding 60 cc., and were collected at intervals, after running out about 25 cc., to insure the collection of circulating blood. The titratable alkali was determined by the method of Greenwald and Lewman (25) and the lactic acid by a slight modification of that of Scott and Flinn (37). This unquestionably gives too high results in normal blood because glucose also yields a substance reacting with iodine but the rise after the injection of alkalies is almost certainly due to lactic acid for almost identical values were then obtained when the blood was precipitated with acid ammonium sulfate solution, the filtrate extracted with ether in a continuous extraction apparatus, and the extract, after evaporating off the ether and alcohol, used for the determination.

Some of the blood was collected under paraffin, centrifuged at once, the cell volume was read off and the plasma used for the determination of hydrogen ion concentration, by Cullen's method (employing the correction 0.18 to bring the pH at 20° to that at 38°), of total and bicarbonate CO_2 by Van Slyke's methods, and of sodium. In the later experiments, this was determined by

Kramer's method but, in Experiments 1 to 7, the plasma was precipitated with picric acid, the filtrate was oxidized with sulfuric and nitric acids, the sulfuric and phosphoric acids were removed with barium hydroxide, this, in turn, with carbon dioxide and, finally, the sodium and potassium were weighed as mixed sulfates, in which the potassium was determined, gravimetrically, as the cobalti-nitrite and the sodium by difference.

The urines secreted during the period of the experiment were analyzed by the following methods: Alkalinity, addition of excess H_2SO_4, boiling to remove CO_2, and titration with NaOH, using methyl red as indicator; chlorine, weighing as AgCl; phosphoric acid, Bell and Doisy (38); potassium and sodium, as in the picric acid filtrate from plasma; occasionally also by Kramer's method; lactic acid, Scott and Flinn (37).

It seems unnecessary to present detailed protocols. Three typical experiments are reported in tabular form and a table comparing the toxicity of sodium carbonate and bicarbonate with that of other sodium salts is presented.

SUMMARY.

After intravenous injection of sodium carbonate or bicarbonate into dogs, there is a retention of carbon dioxide which is so great that the reaction of the blood is changed only slightly. Such change as does occur, may be produced with comparatively small doses. If the injection is made sufficiently slowly to avoid apnea but is continued for a sufficiently long period, convulsions appear. Coincidently, the bicarbonate content and the alkalinity of the blood begin to diminish and this process continues so that, before death occurs, both may be less than normal. The change is due, in part, to the increased production of lactic acid. There is some evidence of the formation of some other non-volatile acid. The production of lactic acid appears to be increased before convulsions appear but it is enormously accelerated thereby.

At the time convulsions appear, the concentration of sodium in the plasma is the same as was observed after the injection of other sodium salts (chloride, sulfate, phosphate). The convulsions are ascribed to "sodium poisoning," a disturbance, due to an excess of sodium, of the normal equilibrium between sodium and other ions.

The evidence in favor of a relation between alkalosis and the tetany of parathyroidectomized dogs, gastric tetany, and the tetany of hyperpnea is examined. It is shown that only in the last named is there any indication of a change in the reaction of the blood and that, even in this case, it is not the alkalosis, *per se*, but the tissue anoxemia that results from the increased stability of the oxyhemoglobin, that is the exciting factor.

I am indebted to Mr. Joseph Gross and to Mr. Max Meyer for their assistance with the experimental work.

BIBLIOGRAPHY.

1. Van Slyke, D. D., *J. Biol. Chem.*, 1921, xlviii, 153.
2. Wilson, D. W., Stearns, T., and Janney, J. H., Jr., *J. Biol. Chem.*, 1915, xxi, 169.
3. Wilson, D. W., Stearns, T., and Thurlow, M. deG., *J. Biol. Chem.*, 1915, xxiii, 89.
4. Wilson, D. W., Stearns, T., and Janney, J. H., Jr., *J. Biol. Chem.*, 1915, xxiii, 123.
5. MacCallum, W. G., and Voegtlin, C., *J. Exp. Med.*, 1909, xi, 118.
6. Berkeley, W. N., and Beebe, S. P., *J. Med. Research*, 1909, xx, 149.
7. Joseph, D. R., and Meltzer, S. J., *J. Pharmacol. and Exp. Therap.*, 1910–11, ii, 361.
8. Greenwald, I., *Am. J. Physiol.*, 1911, xxviii, 103.
9. Greenwald, I., *J. Biol. Chem.*, 1913, xiv, 363.
10. Greenwald, I., *J. Biol. Chem.*, 1913, xiv, 369.
11. Paton, D. N., and Findlay, L., *Quart. J. Exp. Physiol.*, 1916, x, 203.
12. Levy, R. L., Rowntree, L. G., and Marriott, W. McK., *Arch. Int. Med.*, 1915, xvi, 389.
13. Barcroft, J., The respiratory function of the blood, Cambridge, 1914.
14. Hill, A. V., *J. Biol. Chem.*, 1922, li, 359.
15. Hastings, A. B., and Murray, H. A., Jr., *J. Biol. Chem.*, 1921, xlvi, 233.
16. Underhill, F. P., and Nellans, C. T., *J. Biol. Chem.*, 1921, xlviii, 557.
17. Hougardy, A., *Arch. internat. Physiol.*, 1904, i, 17.
18. Scott, R. W., *Am. J. Physiol.*, 1918–19, xlvii, 43.
19. Palmer, W. W., and Van Slyke, D. D., *J. Biol. Chem.*, 1917, xxxii, 499.
20. Howland, J., and Marriott, W. McK., *Quart. J. Med.*, 1917–18, xi, 289.
21. Harrop, G. A., Jr., *Bull. Johns Hopkins Hosp.*, 1919, xxx, 62.
22. Tileston, W., cited by Palmer and Van Slyke (19).
23. Healy, W. P., *Am. J. Obst. and Gynecol.*, 1921, ii, 164.
24. Cullen, G. E., *J. Biol. Chem.*, 1922, lii, 501.
25. Greenwald, I., and Lewman, G., *J. Biol. Chem.*, 1922, liv, 269.
26. Macleod, J. J. R., and Knapp, H. J., *Am. J. Physiol.*, 1918–9, xlvii, 189.
27. Greenwald, I., *J. Pharmacol. and Exp. Therap.*, 1918, xi, 281.

28. Hastings, A. B., Murray, C. D., and Murray, H. A., Jr., *J. Biol. Chem.*, 1921, xlvi, 223.

29. McCann, W. S., *Proc. Soc. Exp. Biol. and Med.*, 1921–22, xix, 393.

30. Collip, J. B., and Backus, P. L., *Am. J. Physiol.*, 1920, li, 568.

31. Grant, S. B., and Goldman, A., *Am. J. Physiol.*, 1920–21, lii, 209.

32. Collip, J. B., *Am. J. Physiol.*, 1920–21, lii, 483.

33. Davies, H. W., Haldane, J. B. S., and Kennaway, E. L., *J. Physiol.*, 1920–21, liv, 32.

34. Bazett, H. C., and Haldane, J. B. S., *J. Physiol.*, 1921, lv, p. iv.

35. Morris, N., *Brit. J. Exp. Path.*, 1922, iii, 101.

36. Hill, L., and Flack, M., *J. Physiol.*, 1910, xl, 347.

37. Scott, E. L., and Flinn, F. B., *J. Biol. Chem.*, 1922, l, p. xxxii.

38. Bell, R. D., and Doisy, E. A., *J. Biol. Chem.*, 1920, xliv, 55.

Note Added to Proof.—Tisdall has recently reported[1] the results of some experiments in which he injected sodium phosphate and phosphoric acid into dogs which he discusses under the title "The influence of the sodium ion in the production of tetany." According to Tisdall, there was no increase in the sodium content of the serum after the injection of Na_2HPO_4, although the inorganic phosphorus of serum was increased by as much as 26 mg. per 100 cc., without any accompanying change in reaction or in CO_2-combining power. What neutralized the phosphoric acid retained in the serum is not evident nor is there any satisfactory explanation for the disappearance of the sodium injected with the phosphorus. It could not have been excreted during the injection, for the author (27) has shown that, during this period, the excretion of sodium is less than the amount equivalent to the phosphorus excreted. Possibly the sodium was excreted in the hour between the close of the injection of the phosphate and the drawing of the blood.

Tisdall's results for the sodium in the serum do not agree with those obtained by the author (27) for sodium in plasma after injections of sodium phosphate. In discussing those experiments, the author presented the hypothesis of "sodium poisoning," a disturbance, due to excess of sodium, of the normal relations between sodium and other ions, to explain the tetany and convulsions observed after injection of sodium phosphate, sulfate, or chloride and, in a later communication,[2] the hypothesis was extended to offer an explanation of the tetany observed after injection of sodium bicarbonate or after hyperpnea, gastric lavage, or persistent vomiting. Tisdall completely neglects to mention either of these papers.

---

[1] Tisdall, F. F., *J. Biol. Chem.*, 1922, liv, 35.

[2] Greenwald, I., *Proc. Soc. Exp. Biol. and Med.*, 1920–21, xviii, 228.

EVALUATION OF BUFFERS OF THE BLOOD.

By EDWARD A. DOISY, A. P. BRIGGS, AND EMILY P. EATON,

(*From the Laboratories of Biological Chemistry, Washington University
School of Medicine, St. Louis.*)

AND WILLIAM H. CHAMBERS.

(*From the Research Laboratories of the Barnard Free Skin and Cancer
Hospital, and the Department of Surgery, Washington University
School of Medicine, St Louis.*)

INTRODUCTION.

In a recent review, Van Slyke (1921) had ably summarized the
rôle of the various blood buffers in the carrying of carbon dioxide.
However, his summary may be looked upon only as a first approxi-
mation because the data upon which his calculations are based
are taken from a variety of sources. For instance, he has made
use of the old determinations of Abderhalden of phosphate in
ox blood for the calculation of the buffer value of the phosphate of
human blood. Furthermore, since there were no suitable data it
has been necessary to assume a normal hemoglobin content for
the two samples of human blood discussed. Likewise the data
of Campbell and Poulton (1920–21) from which Van Slyke has
calculated the buffer value of hemoglobin do not appear to be
very accurate. Some of their points fall on either side of the
curve by a few volumes per cent. It is not surprising then that
we find in Van Slyke's paper (p. 161) the following statement:
" . . . it must be confessed that the data could hardly be
less satisfactory for estimating the relative parts that the buffers
play in the total buffer effect of the blood."

In view of the importance of the subject, we have collected the
data necessary for a recalculation of the degree of participation of
the various buffers in the transport of carbon dioxide. This
work has involved a study of the effect of oxygen unsaturation,

determinations of the carbon dioxide dissociation curves of oxygenated and reduced blood, of separated sera and of dialyzed hemoglobin to which bicarbonate had been added and inorganic phosphate analyses of the blood and serum. The buffer systems studied are identical with those discussed by Van Slyke but in one case our entire data were obtained from a single sample of blood.

<div align="center">PROCEDURE.</div>

The carbon dioxide and oxygen analyses were made by the technique described by Van Slyke and Stadie (1921) and the phosphate analyses by the method of Bell and Doisy as modified by Briggs (1922). The gas mixtures were analyzed with a Henderson burette. Four persons trained in blood gas investigation carried out the various analyses.

The blood which was drawn from the cubital vein was defibrinated by shaking with glass beads in some of our experiments, and oxalated in others. In case a venous sample was desired it was taken with the usual precautions over oxalate after the blood had been flowing from the needle for a few seconds. In our three experiments discussed in detail in this paper it was found necessary to use a little stasis near the end of collection to secure a sufficient quantity of blood. The blood in all of these three experiments was defibrinated.

Three samples of the blood were simultaneously equilibrated at 38° with air or oxygen containing 20, 40, or 60 mm. of pressure of carbon dioxide and three others with hydrogen containing approximately the same tensions of carbon dioxide. Upon completion of the equilibration, the blood was transferred under oil to a small tube without having come in contact with any other gas. Analyses of CO_2 and O_2 in the blood and gas mixture were immediately carried out. The data so obtained were utilized to plot the curves of reduced and oxygenated blood of the three subjects whose bloods were studied.

During the equilibration of the blood another sample of blood was centrifuged to obtain separated serum which was transferred to a paraffined tube and kept at 1°C. until the following day. The carbon dioxide absorption curve of this serum was determined. The data, so obtained on the separated serum, were plotted using

the combined CO_2 on the abscissa and pH on the ordinate.   The curves (Curves 3, 4, and 5) are given in Fig. 1.   From such a chart it is easy to interpolate the change in combined CO_2 for a given change of pH.   The curves demonstrate rather clearly the poor

Fig. 1. Curve 1, hemoglobin solution from blood of W. H. C.; Curve 2, hemoglobin solution from blood of J. M.; Curve 3, separated serum of J. M.; Curve 4, separated serum of E. A. D.; Curve 5, separated serum of W. H. C.

The combined CO_2 is plotted against the pH calculated from the Hasselbalch formula.   The work of Campbell and Poulton indicates that such a calculation is permissible for oxyhemoglobin solutions when the pH is more alkaline than the isoelectric point.

buffer value of the self-possessed serum buffers.   It is obvious that a comparatively large decrease of pH would be necessary for a separated serum to give up alkali sufficient to bind 2 or 3 cc. of CO_2 which is the amount carried in the blood (E.A.D.) showing least difference between arterial and venous states.   The shape

and slope of the curves of the separated sera of the blood of E.A.D.
and W.H.C. show a very pleasing agreement.   The curve of J.M.
differs appreciably from these but whether this is a real difference
or an error in the technique we are unable to state.

The cells which were obtained by centrifuging a sample of blood
were washed four times with isotonic salt solutions, laked with
distilled water, and dialyzed at 1°C. under toluene against many
changes of distilled water and then against water containing suffi-
cient carbon dioxide to give a pH of 4.   After several days, analyses
of the hemoglobin solution showed the presence of only a trace of
inorganic anions and cations.   The solution was then centrifuged
at high speed and the clear supernatant liquid taken off with a
pipette from the thick viscous sediment.[1]   Sodium bicarbonate
was added in sufficient quantity to give a concentration of ap-
proximately 0.03 m.   This solution was equilibrated with various
carbon dioxide-air mixtures and then analyzed for CO_2.   From the
data so obtained curves were plotted with the combined carbon
dioxide on the abscissa and the calculated pH on the ordinate.
The main point of uncertainty in this work was the calculation of
pH because of lack of sufficient reliable data on the solubility
coefficient of CO_2 in hemoglobin solutions.   In a single case
Bohr (1909) has given 0.522 as the solubility coefficient of an
11 per cent solution of hemoglobin and from this and the strength
of our solutions, we have calculated the coefficient used.

The hemoglobin concentration was determined gasometrically
and colorimetrically (Palmer, 1918).   Data on the hemoglobin
prepared from the blood of E.A.D. were discarded because of
lack of agreement in the results of these two methods.   It is
probable that some methemoglobin was present although it was
not positively identified spectroscopically.

[1] Centrifugation actually stratified the solution into three layers; a thin
upper layer which was cream-colored and resembled butter in its consist-
ency; a lower layer which was composed of solid particles which were insolu-
ble in water and dilute alkali; and an intermediate portion which was a
clear deep red solution.   The last named fraction was separated from the
other two and used for our work.   It represents neither completely
purified hemoglobin nor dialyzed cell contents, but we feel that it ap-
proached the former pretty closely.   It is possible and indeed probable
that some of the substances of the two strata which were not used possess
buffer value.   In keeping with this idea is our failure to account for the
entire 100 per cent of the CO_2 which was carried.

The amount of hemoglobin in the solution prepared from blood of W.H.C. was found to be equivalent to 18.0 cc. and that from J.M., 14.0 cc. of O_2 per 100 cc.   We have taken as the CO_2 solubility coefficient for the former 0.515 and for the latter 0.524. These values are probably slightly incorrect, but we do not see how this can introduce any serious error in our deductions.

Study of Curves 1 and 2 of Fig. 1 shows a decided difference in the capacity of the two solutions to furnish base for combination with carbonic acid.   This is to be expected on the ground of the difference in concentration of hemoglobin.   However, if we take, for example, a range of pH from 7.30 to 7.20 and correct the values to the same concentration of hemoglobin we get the results given in Table I.

TABLE I.

Buffer Value of Hemoglobin.

Curve No.	Volumes per cent combined CO_2.			
	At pH 7.30.	At pH 7.20.	Difference.	Corrected to Hb equivalent to 18 volumes per cent O_2.
1	26.3	30.3	4.0	4.00
2	36.8	39.5	2.7	3.47

We can see no valid reason for this discrepancy (4.0 and 3.47 volumes per cent CO_2) but it may be pointed out that the solutions may have differed in their degree of purity.   We are inclined to believe that since we were measuring larger differences in carbon dioxide concentrations, that the data from which Curve 1 are constructed are the more reliable.   This curve was used in the calculations of the buffer value of the hemoglobin of W.H.C. and of E.A.D.; the other for the blood of J.M.

Our data, then, include the carbon dioxide absorption curves of oxygenated blood or reduced blood, of separated serum, of a hemoglobin-bicarbonate solution, and of phosphate analyses of blood and serum upon a single sample of blood (J.M.).   In the case of W.H.C., we have used data obtained from a study of two different samples of his blood.   For E.A.D. all of the data except that of the dialyzed hemoglobin were obtained upon a single sample of his blood.

In addition to the data enumerated, we have determined the O_2 and CO_2 content of one or more samples of venous blood from each of these subjects. This permits us to deal with the probable arterial to venous change in the body.

Relation of Oxygen Unsaturation to Increased Capacity to Bind Carbon Dioxide.

Ordinate Correction (Peters, Barr, and Rule, 1921).—Our first task was an attempt to establish a little more accurately the effect of oxygen unsaturation on the capacity of the blood to bind carbon dioxide. As is already well known, Christiansen, Douglas, and Haldane (1914) were the first to demonstrate clearly that the blood in which the hemoglobin has been reduced binds more CO_2 at any CO_2 tension than when the hemoglobin is present in the oxygenated form. Since their paper, both Parsons (1917) and Joffe and Poulton (1920–21) have published work on oxygenated and reduced blood. The quantitative relationship between the increment of CO_2 and the degree of oxygen unsaturation has been discussed[2] by L. J. Henderson (1921). None of the earlier workers appear to have appreciated the importance of the quantitative relationship and have not given sufficient data on O_2 determination to permit of their results being utilized with certainty.

Peters, Barr, and Rule (1921) in attempting an analysis of this relationship have calculated an empirical factor to correct venous points for oxygen unsaturation, but in so doing have been forced to assume that the hemoglobin content of Haldane's and of Joffe's blood did not vary from day to day. By obtaining complete data on several single samples of blood, we have attempted to make good this deficiency. Most of the experiments were carried out on oxalated blood, but some were on defibrinated blood. Contrary to the findings of Haggard and Henderson (1920–21) and in agreement with Peters, Barr, and Rule (1921), we find that the effect of oxygen unsaturation is quantitatively the same in defibrinated and oxalated blood. This result was first obtained on dog blood and later substantiated in our work on human blood. Results on two specimens of blood from J.M. are given in Table II.

[2] L. J. Henderson has very clearly stated this situation and called attention to the equilibrium existing between the oxygen of the blood and the carbon dioxide of its plasma.

We have taken the liberty of applying the term *ordinate correction* to the type of correction for oxygen unsaturation that Peters, Barr, and Rule have introduced. This terminology is used because the numerical value is obtained by dividing the difference in the ordinates (volumes per cent CO_2) of the curves of reduced and oxygenated blood by the oxygen unsaturation of the hemoglobin of the reduced blood.

The agreement is not exact, but we think that it is as good as could be expected due to the nature of the work.

It will be noted that our values for the correction of oxygen unsaturation are appreciably lower than those given by Peters, Barr, and Rule (0.34). In Table III, we have tabulated the data from all our experiments on human blood in which the equilibration occurred at carbon dioxide tensions which might exist in the body. We have calculated the mean value for the ordinate correction and found it to be 0.27.

TABLE II.

Effect of Oxygen Unsaturation on Oxalated and Defibrinated Blood.

	Oxalated blood.		Defibrinated blood.	
pH of reduced samples	7.35	7.17	7.36	7.21
Ordinate correction	0.25	0.24	0.28	0.27
Isohydric ratio	0.42	0.45	0.47	0.45

Feeling rather confident that we could not have made so gross an error as indicated by the difference between the ratios 0.27 and 0.34, we attempted to analyze the data from which the curves of Joffe and of Haldane were constructed. We found that the carbon dioxide absorption curves were drawn from points determined upon a number of different days. In a few cases sufficient data obtained on a single day were presented which permitted us to calculate the difference in ordinates between an oxygenated and reduced point. Of course, we are making the possibly unjustified assumption that the blood remained unaltered during the period of observation each day. The values obtained from these calculations are given in Table IV.

While the values so obtained are rather variable, it is obvious that our treatment of Haldane's data lends some support to the mean value obtained by us on the study of several samples of

blood.　Parsons' data are more or less neutral while Joffe's data seem to support the value given by Peters, Barr, and Rule.　However, we have given reasons for thinking that the denominator of the fraction $\dfrac{CO_2}{O_2}$ is too small in this case which, if true, would reduce the values to a lower level.

TABLE III.

Ordinate Correction for Oxygen Unsaturation.

Subject. (1)	CO_2 tension of reduced samples. (2)	CO_2 difference between reduced point and oxygenated curve. (3)	O_2 difference in chemically com- bined between reduced and oxygenated blood. (4)	$\dfrac{CO_2}{O_2}$ Values of Column 3 divided by values of Column 4. (5)
	mm.	*vol. per cent*	*vol. per cent*	
J. M. K.	33.7	5.0	18.0	0.28
	60.3	1.6	13.0	0.12*
M. D.	37.0	4.7	17.7	0.25
	61.3	5.4	17.4	0.29
G. D.	32.0	5.2	18.4	0.28
	63.8	3.6	18.6	0.20
J. M.	37.5	4.7	18.8	0.25
	68.4	4.5	18.7	0.24
M. H.	70.5	5.0	16.5†	0.30
M.	34.7	4.4	16.0	0.28
E. A. D.	46.3	4.3	15.5	0.28
W. H. C.	42.5	4.3	14.5	0.30
	83.3	3.8	14.8	0.26
J. M.	37.7	4.7	17.4	0.27
	61.7	4.5	16.5	0.27
E. A. D.	37.5	5.2	18.3†	0.28
	71.6	5.4	18.3†	0.29
W. H. C.	35.2	4.4	15.5†	0.28
	63.8	4.5	15.5†	0.29
Mean of first 12 results..........................				0.268
"　　" last 6 results.............................				0.283
"　　" 18 results.................................				0.272

* Omitted in taking the mean.

† O_2 content of reduced blood calculated from Hill's equation.

In the lower section of Table III, we have tabulated our results on the three samples of blood which are to be discussed in detail later.　It is evident that the agreement with our mean values is very good in the blood of J.M. but not so pleasing in the other two samples.　We consider it more justifiable to use the mean

TABLE IV.

Ordinate Correction: Data from Blood of Haldane, Parsons, and Joffe.

Subject.	CO_2 tension of reduced samples.	CO_2 difference between reduced point and oxygenated curve.	O_2 difference in chemically combined between reduced and oxygenated blood.	$\dfrac{CO_2}{O_2}$ Values of Column 3 divided by values of Column 4.
(1)	(2)	(3)	(4)	(5)
	mm.	*vol. per cent*	*vol. per cent*	
Haldane.	78.7	9.0	18.0	0.50
	37.9	4.9	18.0	0.27
	58.2	4.9	18.0	0.27
	74.2	5.0	18.0	0.28
	34.5	3.3	18.0	0.18
	60.9	5.4	18.0	0.30
Parsons.	19.6	4.3	18.0	0.24
	33.4	6.3	18.0	0.35
	37.4	3.5	18.0	0.20
	72.1	3.4	18.0	0.19
Joffe.	27.6	5.3	16.8	0.32
	36.8	6.6	16.8	0.39
	50.5	5.5	16.8	0.33
	41.7	1.6	16.8	0.10
	47.0	5.0	16.8	0.30
	55.7	5.4	16.8	0.32
	82.2	9.2	16.8	0.55

In our consideration of the bloods of Haldane, Parsons, and Joffe, we are confronted with doubt as to the value of unsaturation that should be used. We have assumed that the concentration of hemoglobin on the different experimental days was constant and since no data on this point are given in the papers of Haldane and of Parsons that it was equivalent to 18.5 volumes per cent of oxygen from which we deduce the oxygen unsaturation to be about 18.0 cc. We are more fortunate in the data given on Joffe's blood in that the oxygen capacity is stated to be 18.7 volumes per cent, but we do not know whether this value holds for all experimental days. Assuming that it does the oxygen unsaturation was about 16.8 cc.

We question the application of the oxygen capacity value given by Joffe for two reasons: (1) The absorption curve of his oxygenated blood rises a little more rapidly than those of the different samples of blood containing about 18.0 volumes per cent of oxygen that we have studied; and (2) The corpuscle volume is generally roughly parallel to the amount of hemoglobin present. We have found that there is more than the normal oxygen capacity when the corpuscular volume exceeds that of the serum. The mean of Joffe's hematocrit determinations gives the volume of corpuscles as 51 per cent. A blood with corpuscle volume of 40 to 45 per cent generally has an oxygen capacity of 18 to 19 cc. per 100 cc.

Data were taken from the following papers in the preparation of this table: Christiansen, Douglas, and Haldane (1914), p. 249; Parsons (1917), p. 448; Joffe and Poulton (1921–22), Table VII, p. 150.

313

value, 0.27, to correct for the effect of oxygen unsaturation; *i.e.*, an average value possesses more weight than the individual determinations.

Isohydric Correction for Oxygen Unsaturation.

When additional CO_2 is taken up by blood *without change in pH* an increase in oxygen unsaturation must result. The ratio between the volume of CO_2 gained and of O_2 lost, without change in pH, we have designated the "isohydric correction for oxygen unsaturation." This factor necessarily cannot be a constant because the acid dissociation constants of oxy- and reduced hemoglobin are slightly different but it is approximately so over the small range of pH considered in our paper.

The total CO_2 values of the oxygenated and reduced bloods have been plotted against their calculated pH and the isohydric increase of total CO_2 read from the charts. Oxygen was determined in the oxygenated and reduced specimens and the data obtained in our studies are given in Table V. The mean value was found to be 0.44 which means that when 1 cc. of combined O_2 is lost 0.44 cc. of CO_2 must be added to maintain the pH without change. When 1 cc. of O_2 is lost at any normal CO_2 tension some base is freed to combine with CO_2 which increases the bicarbonate and would make the blood more alkaline were not the CO_2 tension increased to compensate this (Henderson, 1921).

Evaluation of the Buffers of the Blood.

As Van Slyke has pointed out in his review of the carbon dioxide carriers, there are two distinct types of buffer action in the blood:

1. That due to the reduction of hemoglobin, *i.e.* when the arterial blood loses oxygen in its passage through the capillaries, a certain definite quantity of carbon dioxide must be taken up with an increase in bicarbonate and the partial pressure of CO_2 must be raised to maintain the pH without change and to prevent the blood from actually becoming more alkaline. This may be called an *isohydric* increase of carbon dioxide and according to the present theory it is due entirely to hemoglobin being a weaker acid than oxyhemoglobin.

2. That due to still further increase in the concentration of H_2CO_3 which means an increase in hydrogen ion concentration which naturally causes a readjustment of the various buffer systems by which some of the alkali of the salts of the various

TABLE V.

Isohydric Absorption of Carbon Dioxide (Total CO_2) with Loss of Oxygen.

Subject.	pH of reduced samples.	Isohydric difference of CO_2.	O_2 unsaturation of hemoglobin.	Isohydric ratio $\frac{CO_2}{O_2}$.
		vol. per cent	vol. per cent	cc.
J. M. K.	7.40	8.2	18.0	0.46
	7.20	2.4	13.0	0.20*
M. D.	7.35	8.0	17.7	0.45
	7.20	7.8	17.4	0.45
G. D.	7.39	10.0	18.4	0.54
	7.20	6.7	18.6	0.36
E. A. D.	7.32	6.2	15.5	0.40
J. M.	7.35	7.8	18.8	0.42
	7.17	8.3	18.7	0.45
W. H. C.	7.33	6.0	14.5	0.41
	7.12	6.2	14.8	0.42
M. H.	7.18	7.2	16.5†	0.44
M.	7.34	6.7	16.0	0.41
Mean (12 results)				0.433
E. A. D.	7.35	8.5	18.3†	0.46
	7.17	9.3	18.3†	0.51
J. M.	7.36	7.9	17.4	0.45
	7.21	7.6	16.5	0.46
W. H. C.	7.39	7.5	15.5†	0.48
	7.21	6.8	15.5†	0.44
Mean (6 results)				0.466
General mean				0.444

* Excluded from the average.

† Oxygen content of the reduced samples was calculated from Hill's equation.

weak acids is combined with carbon dioxide thereby increasing the concentration of bicarbonate and of the weak acids, including carbonic. In the following systems the numerator is decreased and the denominator increased:

$$\frac{BHbO_2}{HHbO_2} \; ; \; \frac{BHb}{HHb} \; ; \; \frac{B - protein}{H - protein} \; ; \; \frac{B_2HPO_4}{BH_2PO_4}$$

To illustrate more clearly the evaluation of the buffers, we give the details of the calculations of the participation of each in the blood of J.M. when it changes from the arterial to venous condition. In this connection it is necessary to say that the venous blood was analyzed but that no analyses of arterial blood were made. The data of the arterial point are based upon an hypothetical ideal relationship to the venous blood.

Determination of Arterial and Venous Points.

Venous Point.—The venous point on the CO_2 dissociation curve must lie somewhat above the oxygenated curve because of the larger CO_2-combining power at the prevailing CO_2 tension resulting from its oxygen unsaturation. Using our factor of 0.27 volumes per cent of oxygen unsaturation, we have: Combined O_2 of fully saturated blood (19.4) − combined O_2 in venous blood (11.8) = 7.6 volumes per cent venous O_2 unsaturation of hemoglobin. $7.6 \times 0.27 = 2.05$ volumes per cent CO_2 higher than oxygenated curve. (See Fig. 2.)

The observed total CO_2 of venous blood, 53.9 cc. − 2.05 = 51.85 cc., therefore gives the ordinate (volumes per cent CO_2) of a point on the oxygenated curve having the same CO_2 tension (abscissa) as venous blood. A line drawn from the ordinate 51.85 cuts the oxygenated curve at the point corresponding to 59.8 mm. of CO_2 tension. The venous point, therefore, is located at the intersection of 53.9 volumes per cent CO_2 and 59.8 mm. of CO_2.

Its pH is calculated by the familiar Hasselbalch (1917) equation in the form used by Van Slyke (1921).

$$\begin{aligned}
pH &= pK_1 + \log \frac{\text{total } CO_2 - 0.0672 \, p}{0.0672 \, p} \\
&= 6.10 + \log \frac{53.9 - (0.672 \times 59.8)}{0.0672 \times 59.8} \\
&= 7.194 \text{ venous pH}
\end{aligned}$$

Arterial Point.—True arterial blood was not analyzed, but the arterial point can be calculated as follows. Assuming the arterial

Fɪɢ. 2. Blood of J. M. Volumes per cent of total CO_2 are plotted against millimeters of pressure of CO_2. Three points on the oxygenated and reduced curves were determined on the same sample of blood. The reduced point at 37.7 tensions of CO_2 has a greater oxygen unsaturation than the other two reduced points. Consequently we have drawn the curve through a point at a level which corrects for this difference in unsaturation.

blood to be 95 per cent saturated with oxygen (Van Slyke and Stadie, 1921), its oxygen content would be $19.4 \times 0.95 = 18.43$ $+ 0.24$ cc. dissolved oxygen $= 18.67$ volumes per cent O_2 content.[3] The venous blood contained 11.9 volumes per cent O_2; and $18.67 - 11.9 = 6.77$ cc. of O_2 were lost from the blood during its passage through the capillaries. Assuming a normal fasting respiratory quotient[4] of 0.75, the venous blood must have taken up $(6.77 \times 0.75) = 5.08$ cc. of CO_2, and the content

[3] In our discussion of the isohydric absorption of CO_2, we have used the combined O_2 as a measure of the concentration of hemoglobin. Consequently, in the location of the venous point it has been necessary to correct for the volumes per cent of unsaturation of the hæmoglobin. However, in calculating the CO_2 carried from the difference of oxygen content of the arterial and venous blood the actual loss of oxygen (i.e. difference in their oxygen contents) must be considered.

[4] It is obvious that in assuming a respiratory quotient of this value our discussion emphasizes the quota of CO_2 carried isohydrically. If we had assumed a respiratory quotient of 1.00 then the absolute amount of CO_2

of arterial blood was $53.9 - 5.08 = 48.82$ cc. Using 48.82 volumes per cent total CO_2 as the ordinate and allowing for the unsaturation of the arterial blood, the arterial point is located on the chart and its abscissa (mm. of tension) found to be 50.0 mm. of CO_2. The figures for the arterial and venous blood of J.M. are given in Table VI.

TABLE VI.

Arterial and Venous Blood of J. M.

	Venous.	Arterial.
Total CO_2, *vol. per cent*	53.9	48.82
CO_2 tension, *mm*	59.8	50.0
pH	7.194	7.231

Buffer Effect of Loss of Oxygen from Arterial Blood: Type 1.

Our consideration of the first type of buffer reaction (p. 320) has led us to adopt 0.44 as the isohydric increase of CO_2 for every volume per cent of O_2 unsaturation of the blood. The difference in the oxygen unsaturation of the hemoglobin in the arterial and venous blood of J.M. is $18.43 - 11.80 = 6.63$ cc.

$6.63 \times 0.44 = 2.92$ cc. of CO_2 absorbed without change of pH

Starting with 48.82 cc. as the arterial total CO_2, we add to it 2.92 cc. of total CO_2 which were taken up isohydrically by the loss of 6.63 cc. of O_2 from the hemoglobin. From this value and the arterial pH, we can calculate the dissolved CO_2 added in volumes per cent.

$$7.231 = pK_1 + \log \frac{51.74 - 0.0672\,p}{0.0672\,p}$$
$$(13.52 \times 0.0672\,p) + 0.0672\,p = 51.74$$
$$0.9085\,p + 0.0672\,p = 0.9757\,p = 51.74$$
$$p = 53.03 \text{ mm.}$$

carried by a given reduction of hemoglobin would remain the same (isohydric constant, 0.44) but the absolute amounts carried by the buffers coming into play with a change of pH would be greater and there would necessarily be a larger difference in the pH of the arterial and venous blood.

As a nearly related fact, it is necessary to point out the theoretical dependence of the difference of pH between the arterial and venous conditions upon the quantity of oxygen lost in the capillaries and the respiratory quotient. If more oxygen is lost then the change of pH is greater (see Table VII).

But the venous tension was 59.8 mm., so $59.8 - 53.0 = 6.8$ mm. of CO_2 increase in tension to change pH from 7.231 to 7.194 and $53.0 - 50.0 = 3.0$ mm. to maintain arterial pH in the venous blood for the amount of oxygen lost. $3.0 \times 0.672 = 0.20$ cc. of CO_2 physically dissolved. The combined CO_2 then is $2.92 - 0.20 = 2.72$ cc. carried isohydrically by loss of 6.63 cc. of O_2 from the hemoglobin.

Buffer Reactions of Type 2 (Change in pH).

Hemoglobin.—Proceeding to a study of the rôle of the other buffers, reference to the chart of the hemoglobin prepared from J.M.'s blood shows that between the range of pH 7.25 to 7.15, a difference of 0.10, the hemoglobin gave up alkali sufficient to bind 2.7 cc. of CO_2. But the change from arterial to venous was only pH 7.231 to 7.194, or 0.037.

$$2.7 \times \frac{0.037}{0.100} = 0.999 \text{ cc. of } CO_2 \text{ bound by this change of pH}$$

The prepared solution had a hemoglobin content equivalent to 14.0 volumes per cent of O_2 and the blood of J.M. a hemoglobin content of 19.4 volumes per cent of O_2.

$$0.999 \times \frac{19.4}{14.0} = 1.384 \text{ cc. of } CO_2 \text{ by the same change of pH in}$$

the concentration existing in the blood of J.M. This involves the assumption that reduced hemoglobin has the same buffer value as oxyhemoglobin in this range of pH. From some of our work on dog hemoglobin we believe that this is approximately true and that this cannot introduce any appreciable error.

Separated Serum Buffers.—Referring to the separated serum chart, we can read off directly the quantity of combined CO_2 carried by a change of pH from 7.25 to 7.15. It is found that 0.70 cc. of CO_2 is bound by base yielded by separated serum buffers in changing from pH 7.25 to 7.15 and from this it is calculated that 0.259 cc. is yielded in changing from pH 7.231 to pH 7.194. Since this is the quantity yielded per 100 cc. of serum and there are only 55 cc. of serum per 100 cc. of blood, this value must be corrected as follows:

$0.259 \times 0.55 = 0.142$ cc. of CO_2 carried by all buffers of separated serum per 100 cc. of blood.

Phosphates.—Analyses showed the concentration of inorganic phosphate to be 0.00073 m, from which we derived the quantity of base given up by B_2HPO_4 in changing the pH of this system from 7.231 to 7.194 (see Van Slyke (1921), p. 149).

$$pH = pK_1 + \log \frac{Na_2HPO_4}{NaH_2PO_4}$$

$$7.231 = 6.8 + \log \frac{0.00073 - x}{x} \qquad 7.194 = 6.8 + \log \frac{0.00073 - x}{x}$$

$$0.431 = \log \frac{0.00073 - x}{x} \qquad\qquad 0.394 = \log \frac{0.00073 - x}{x}$$

$$2.698 \, x = \frac{0.00073 - x}{x} \qquad\qquad 2.477 \, x = 0.00073 - x$$

$$x = 0.000197 \text{ m} \qquad\qquad\qquad x = 0.000210 \text{ m}$$

$0.000210 - 0.000197 = 0.000013$ m base from B_2HPO_4

$0.000013 \times 2,240 = 0.029$ cc. of CO_2 carried by base from inorganic phosphate buffer system of whole blood.

According to a recent paper by Zucker and Gutman (1922) the concentration of phosphate of serum and of whole blood in fresh specimens is identical. Relying upon this observation with which some of our work coincides, we have made a correction of the phosphate buffer value of serum in order to avoid a duplication.

55 per cent of the blood of J. M. was serum and therefore possessed 55 per cent of the buffer value of the inorganic phosphate which must be subtracted from the total.

$0.029 \times 0.55 = 0.016$ cc. of CO_2 carried by base yielded by phosphate of serum and therefore taken into account in the buffers of separated serum.

$0.029 - 0.016 = 0.013$ cc. of CO_2 carried by the inorganic phosphate of the cells.

Physically Dissolved Carbon Dioxide.—We have now accounted for all of the bound carbon dioxide due to our different known buffer systems. There remains then only the physically dissolved fraction. The total increase is merely the difference of arterial and venous CO_2 tensions multiplied by the solubility coefficient of CO_2 in blood.

$9.8 \times 0.0672 = 0.658$ cc. carried as physically dissolved.

The data of this blood and of the other two studied are tabulated in Table VII, and in Table VIII we have collected the calculated amounts of CO_2 carried by the various buffer systems.

TABLE VII.

Data on Arterial and Venous Blood of Subjects Studied.

	E.A.D	W.H.C.	J.M.
Total O_2 content, arterial blood, *vol. per cent*	18.10	15.44	18.67
" " " venous " " " "	15.00	9.80	11.90
Difference	3.10	5.64	6.77
Oxygen capacity of hemoglobin, *vol. per cent*	18.80	16.0	19.40
Chemically combined O_2, arterial blood, *vol. per cent*	17.86	15.20	18.43
" " " venous " " " "	14.87	9.70	11.80
Difference	2.99	5.50	6.63
Total CO_2, arterial blood, *vol. per cent*	46.58	50.27	4?.82
" " venous " " " "	48.90	54.50	53.90
Difference	2.32	4.23	5.08
Dissolved CO_2, arterial blood, *vol. per cent*	3.10	3.25	3.36
" " venous " " " "	3.35	3.76	4.02
$BHCO_3$, arterial blood, *vol. per cent*	43.48	47.02	45.46
" venous " " " "	45.55	50.74	49.88
CO_2 tension, arterial blood, *mm.*	46.2	48.4	50.0
" " venous " "	49.9	56.0	59.8
Difference	3.7	7.6	9.8
pH, arterial blood	7.246	7.260	7.231
" venous "	7.233	7.230	7.194
Difference	0.013	0.030	0.037
Total CO_2, isohydric absorption, *vol. per cent*	1.320	2.42	2.92
H_2CO_3 " " " " "	0.087	0.158	0.20
$BHCO_3$ " " " " "	1.233	2.262	2.72

TABLE VIII.

Carbon Dioxide Carried by Buffer Systems Studied.

	E.A.D.		W.H.C.		J.M.	
	vol. per cent	per cent of total	vol. per cent	per cent of total	vol. per cent	per cent of total
Total CO_2 carried for R. Q. of 0.75..	2.32		4.23		5.08	
$BHCO_3$ carried isohydrically						
$BHbO_2 \rightarrow HHb$...................	1.233	53.1	2.262	53.5	2.72	53.5
$BHCO_3$ carried by change of pH.						
By hemoglobin: $BHbO_2 \rightarrow HHbO_2$						
$BHb \rightarrow HHb$....	0.439	18.9	1.070	25.3	1.384	27.2
By B_2HPO_4 in cells..............	0.010	0.43	0.012	0.3	0.013	0.25
By separated serum............	0.089	3.84	0.198	4.7	0.142	2.8
CO_2 physically dissolved...........	0.249	10.7	0.511	12.1	0.657	12.9
Sum, *per cent of total*..............	2.020	87.0	4.053	96.0	4.196	97.0
Per cent of total CO_2 carried by						
hemoglobin.....................		72.0		78.8		80.7

DISCUSSION OF RESULTS.

From our data, we have calculated the relative amount of alkali furnished by each of the various known blood buffers for binding carbon dioxide under a normal arterial to venous change of state. The percentage of increment of total carbon dioxide accounted for in the three samples studied varies from 87 to 97. With regard to the experiment in which only 87 per cent was accounted for, it may be stated that the discrepancy apparently lies in that the calculated pH change is too small. By the manner in which we determine the isohydric quota of CO_2 it is to be expected that these results for the three samples of blood would agree. However, the CO_2 carried by the change in reaction of this blood is 10 per cent less than that of the other two. This may mean an error in our pH calculation which is possibly due to a mistake in the data or in construction of the oxygenated carbon dioxide absorption curve. It might also be referred to a lack of constancy of the isohydric $\frac{CO_2}{O_2}$ ratio over the range studied; reference to Table V shows that this blood gave a ratio appreciably higher than the other two and the average figure used in our calculations. This latter possibility must be strongly discounted

if our present idea of the effect of reduction is to remain unmodified. In this connection it may be noted that the isohydric ratios found with oxygenated and reduced dialyzed hemoglobin are generally considerably lower than those obtained for blood. We hope to be able to elucidate this point at a later date.

The arterial to venous pH change requires some comment. Both Hasselbalch and Parsons consider this change to be about 0.02. Parsons in particular has based this estimate on the loss of 6.0 cc. of O_2 and the taking up of sufficient CO_2 to give a normal respiratory quotient. As we have studied his paper it appears to us that his data are not adequate to make this approximation conclusive. The results from which his curves are constructed are quite scattered, which may be due to his utilization of data obtained on blood on different experimental days, and we have not been able to use them in our study.

Still more recently Peters, Barr, and Rule have studied the difference of arterial and venous pH of normal men and have found it to be less than 0.02. While we appreciate the great technical difficulties[5] in work of such a nature, we must call attention to two points both of which would cause a greater change of pH than that calculated by these investigators.

1. The factor (0.34) used by them to correct for oxygen unsaturation based on data on the blood of Haldane and of Joffe is appreciably greater than that found by us (0.27) in our work. This would give a greater quota of carbon dioxide taken up isohydrically due to the oxygen unsaturation and lessen the difference in CO_2 tension between the arterial and venous blood.

2. In most of their experiments and in all of the normal cases studied low blood respiratory quotients were found. This means that in the utilization of their data, they have corrected for a greater degree of oxygen unsaturation with respect to CO_2 carried than could probably exist physiologically for any length of time, and have thereby found changes of pH which are too small. At the present time it seems very probable to us that generally the blood at rest must have a normal respiratory quotient and in

[5] This point has been discussed by Peters, Barr, and Rule (p. 515). We might add that to our knowledge, no one has been uniformly successful in obtaining normal respiratory quotients in a study of arterial and venous blood of men.

fact we have found this to be true in blood drawn simultaneously from the artery and vein of the same leg of a dog in several experiments.

To satisfy ourselves, we have recalculated from the data given by Peters, Barr, and Rule the probable change of pH when allowance is made for these two points. To do this we have used their oxygen values and arterial CO_2 value and assumed that sufficient CO_2 must have been added to the arterial blood in becoming venous to give a normal respiratory quotient of 0.75. With such assumptions and using the blood of D.P.B. whose respiratory quotient (0.67) is the closest to normal the difference between the calculated arterial and venous pH is about 0.055 which is much greater than the difference indicated by the position of the arterial and venous points on their charts.

This, then, seems to explain the difference between their conclusions and ours on the arterial to venous pH changes. Theirs are based on actual determinations on arterial and venous blood showing physiologically improbable respiratory quotients; ours on venous blood and an ideal respiratory quotient.

It might be well to recall that in Van Slyke's calculations of the buffers of Haldane's blood that for the loss of 6.3 cc. of O_2 and the respiratory quotient of 0.75, the change of pH is 0.03.

It seems worth while to point out that our calculated differences in arterial and venous CO_2 tensions in the bloods of W.H.C. and J.M. are also a little larger than the usual estimate. In the former it is 7.6 mm. and in the latter 9.8 mm. Both Haldane and Parsons estimate it to be from 5 to 7 mm., but Peters, Barr, and Rule have experimentally found differences as large as 10 mm. with an average of 6 mm.

Our results for the venous pH appear to be more acid than those generally accepted. They have been calculated from the Hasselbalch formula which Donegan and Parsons (1918–19) have shown to give pH values which are less by about 0.07 than those obtained by them with the hydrogen electrode, which measures the pH of the plasma. We are inclined to agree with them on this score since our calculated values on blood and its serum show a similar difference. Our results on subject E.A.D. are given in Table IX for comparison.

Fig. 3. Blood of E. A. D. Volumes per cent of total CO_2 are plotted against millimeters of pressure of CO_2. Three points on the oxygenated and reduced curves were determined on the same sample of blood.

Fig. 4. Blood of W. H. C. Volumes per cent of total CO_2 are plotted against millimeters of pressure of CO_2. The data were obtained from two samples of blood drawn at an interval of 6 weeks.

The mean difference seems to indicate that the calculated pH of the serum exceeds that of the blood by 0.05 +. The use of 6.15 as pK_1 for blood and 6.10 for serum as advocated by Van Slyke (1922) would cause this difference to disappear. Possibly it might have been advisable to use this value in our calculations, but the various curves have so nearly the same slope over a longer range that no appreciable error has been introduced. It may well be that the pH of our venous samples was 0.05 greater than the figures given in the text, which would give us the values 7.244, 7.28, and 7.283. The absolute values depend upon the value accepted for pK_1.

TABLE IX.

Calculated pH of Blood and Its Serum.

	Oxygenated.			Reduced.		
CO_2, mm	23.3	43.0	72.5	22.4	37.5	71.6
pH serum.......................	7.47	7.33	7.17	7.54	7.41	7.21
pH blood.......................	7.42	7.26	7.12	7.48	7.35	7.17
Difference of pH...............	0.05	0.07	0.05	0.06	0.06	0.04

SUMMARY.

In a study of the various *known buffer* systems of the blood, we have been able to account for 87 to 97 per cent of the carbon dioxide carried in the change from an hypothetical arterial to an actual venous state. As previously pointed out (Van Slyke, 1921), the hemoglobin plays the preeminent rôle in the transport of both carbon dioxide and oxygen. About 75 to 80 per cent of the carbon dioxide carried is due to the hemoglobin, the remainder being carried by the other buffer systems. The buffer value of the inorganic phosphate is shown to be less than 1 per cent of the total, while that of the separated serum is less than 5 per cent. A revised value of 0.27 as a factor to correct for oxygen unsaturation in interpolating venous points is given. The isohydric absorption of 0.44 volumes per cent of carbon dioxide occurs with each volume per cent of oxygen unsaturation of the hemoglobin of the blood.

The authors desire to express their appreciations of Dr. P. A. Shaffer's advice and encouragement in this work; of Dr. C. M.

Gruber's assistance in taking the samples of blood; and of Dr. Michael Somogyi's help in the preparation of the charts. Thanks are due to certain students of the first year class for furnishing blood specimens.

Protocols.

Samples of blood were simultaneously equilibrated at $38°$ with three CO_2 tensions in the practical absence of oxygen and with three in the presence of air or oxygen. The samples were transferred to a small tube under a layer of oil without coming into contact with any other gas. CO_2 analyses were made as rapidly as possible.

The oxygen contents of the reduced samples of blood from E. A. D. and W. H. C. (Dec. 21) were calculated from Hill's equation, but determined in the blood from J. M. and of W. H. C. (Feb. 2).

The separated serum was kept in a paraffined tube over night at $1°C$. Equilibrations and analyses were carried out on the following day.

Both oxygen and carbon dioxide were determined by the methods described by Van Slyke; and in the case of the blood of J. M. and W. H. C. (Feb. 2) by the more recent procedure of Van Slyke and Stadie. Phosphorus was determined colorimetrically by a method described by Briggs (1922).

Subject J. M.

	Oxygenated blood.			Reduced blood.		
CO_2, mm	19.1	37.8	62.1	21.0	37.7	61.7
Total CO_2, vol. per cent	31.2	43.4	52.6	37.2	48.4	57.0
pH	7.47	7.31	7.16	7.50	7.36	7.21
O_2, chemically combined, cc	19.4			2.80	2.0	2.8

	Venous blood
CO_2 content, vol. per cent	53.9
O_2 " " " "	11.9

	Separated serum.		
CO_2, mm	16.3	39.1	60.3
$BHCO_3$, vol. per cent	45.5	52.1	53.5
pH	7.69	7.37	7.20

	Hemoglobin-bicarbonate solution.			
CO_2, mm	18.0	29.9	44.2	60.9
$BHCO_3$, vol. per cent	32.7	36.1	39.0	42.4
pH	7.52	7.34	7.21	7.10

Hemoglobin equivalent to
{ 14.0 vol. per cent chemically combined O_2, colorimetrically.
13.8 vol. per cent chemically combined O_2, gasometrically.

Inorganic P in blood, 2.27 mg. per 100 cc.

Subject E. A. D.

	Oxygenated blood.			Reduced blood.		
CO_2, *mm*............................	23.3	43.0	72.5	22.4	37.5	71.6
Total CO_2, *vol. per cent*..........	34.7	45.0	56.0	37.8	47.5	61.2
pH...............................	7.42	7.26	7.12	7.48	7.35	7.17
O_2, *mm*............................	667.00	653.00	622.00	6.4	5.6	5.6
O_2, chemically combined, *cc*......	18.8	18.8	19.1	Approximately 0.50 cc.		

	Serum from oxygenated blood.			Serum from reduced blood.		
Total CO_2, *vol. per cent*.	40.9	55.4	66.4	46.0	57.0	70.6
pH..............................	7.47	7.33	7.17	7.54	7.41	7.21

	Venous blood.
CO_2 content, *vol. per cent*...................................	48.9
O_2 " " " "	15.0

	Separated serum.		
CO_2, *mm*.........................	18.1	41.2	71.2
$BHCO_3$, *vol. per cent*.............	51.3	55.8	58.4
pH...............................	7.70	7.38	7.16

Inorganic P in blood, 5.3 mg. per 100 cc.

Subject W. H. C.

Dec. 21.	Oxygenated blood.			Reduced blood.		
CO_2, *mm*........................	23.0	40.3	67.2	16.7	35.2	63.8
Total CO_2, *vol. per cent*..........	36.0	45.3	56.1	34.4	48.2	59.6
pH...............................	7.45	7.30	7.16	7.57	7.39	7.21
O_2, *mm*........................	661.00	661.00	642.00	6.7	6.6	7.2
O_2, chemically combined, *cc*......	16.0			Approximately 0.50 cc.		

	Venous blood.
CO_2 content, *vol. per cent*...................................	54.5
O_2 " " " "	9.8

	Separated serum.		
CO_2, *mm*.........................	22.7	40.6	63.2
$BHCO_3$, *vol. per cent*.............	57.2	60.3	62.5
pH...............................	7.65	.7.42	7.24

Subject W. H. C.—Continued.

	Hemoglobin-bicarbonate solution.		
CO_2, mm	8.4	28.3	42.4
$BHCO_3$, vol. per cent	16.4	27.9	32.4
pH	7.56	7.26	7.15

Hemoglobin equivalent to $\begin{cases} 18.1 \text{ vol. per cent chemically combined } O_2, \\ \text{gasometrically.} \\ 17.9 \text{ vol. per cent chemically combined } O_2, \\ \text{colorimetrically.} \end{cases}$

Inorganic P in blood, 2.8 mg. per 100 cc.

Feb. 2.	Oxygenated blood.		Reduced blood.*	
CO_2, mm	42.2	76.5	42.5	83.3
Total CO_2, vol. per cent	47.5	58.3	51.9	63.9
pH	7.30	7.11	7.33	7.12
O_2, chemically combined, cc.	18.0		3.5	3.2

* It will be noted that the degree of unsaturation of this reduced blood is practically the same as that of the reduced blood of Dec. 21. A smooth curve can be drawn through all of these reduced points, yet the blood of Dec. 21 was defibrinated and that of Feb. 2, oxalated.

The venous blood of Dec. 21 was used in our calculations.

BIBLIOGRAPHY.

Bohr, C., in Nagel, W., Handbuch der Physiologie des Menschen, Brunswick, 1909, i, 62.

Briggs, A. P., *J. Biol. Chem.*, 1922, liii, 13.

Campbell, J. M. H., and Poulton, E. P., *J. Physiol.*, 1920–21, liv, 152.

Christiansen, J., Douglas, C. G., and Haldane, J. S., *J. Physiol.*, 1914, xlviii, 244.

Donegan, J. F., and Parsons, T. R., *J. Physiol.*, 1918–19, lii, 315.

Haggard, H. W., and Henderson, Y., *J. Biol. Chem.*, 1920–21, xlv, 215.

Hasselbalch, K. A., *Biochem. Z.*, 1917, lxxviii, 112.

Henderson, L. J., *J. Biol. Chem.*, 1920, xli, 401.

Henderson, L. J., *J. Biol. Chem.*, 1921, xlvi, 411.

Joffe, J., and Poulton, E. P., *J. Physiol.*, 1920–21, liv, 129.

Palmer, W. W., *J. Biol. Chem.*, 1918, xxxiii, 119.

Parsons, T. R., *J. Physiol.*, 1917, li, 440.

Peters, J. P., Jr., Barr, D. P., and Rule, F. D., *J. Biol. Chem.*, 1920–21, xlv, 489.

Van Slyke, D. D., *Physiol. Rev.*, 1921, i, 141.

Van Slyke, D. D., *J. Biol. Chem.*, 1922, l, p. xvi.

Van Slyke, D. D., and Stadie, W. C., *J. Biol. Chem.*, 1921, xlix, 1.

Zucker, T. F., and Gutman, M. B., *Proc. Soc. Exp. Biol. and Med.*, 1922, xix, 169.

IS ASPHYXIA THE CAUSE OF DRUG HYPERGLYCEMIAS?

By A. L. TATUM and A. J. ATKINSON.

*(From the Laboratory of Physiological Chemistry and Pharmacology,
University of Chicago, Chicago.)*

It is well known that asphyxia causes glycogenolysis. There-
fore, a number of workers have attributed the hyperglycemia
produced by ether and epinephrine solely to asphyxia. Stewart
in his Manual of physiology states:

"It has usually been assumed that the hyperglycemia of asphyxia is due
to the effect upon the center of blood over-rich in carbon dioxide (and
other metabolic products) or impoverished as regards oxygen."

He also states that there is some evidence that the altered
blood may also affect the liver cells directly by interference with
the internal respiration of the hepatic tissue, operating, it may
be, through an increase in the concentration of the hydrogen
ions (1). Sansum and Woodyatt (2) found that ether and nitrous
oxide increase the glycosuria and D:N ratio in phlorhizin diabetes,
sugar being derived from glycogen "probably through tissue
asphyxia." Ross and McGuigan (3) claim a direct action of ether,
and that asphyxia is not essential. Ritzmann (4) supported the
asphyxial explanation of the glycogenolytic activity of epinephrine
by considering that epinephrine produces a portal stasis (by portal
vasoconstriction) sufficient to cause anoxemia. It has, however,
been shown by one of us (5) that there is no demonstrable degree
of portal 'stasis following subcutaneous injections of epinephrine.

The asphyxial theory of drug hyperglycemia seems to have
been accepted by many investigators with no further proof than
that asphyxia is of itself capable of causing hyperglycemia and
that asphyxia is probably caused by these drugs. This to our
mind leaves open the possibilities of asphyxia and glycogenolysis
being parallel and not interdependent phenomena or that asphyxia
is one of several factors producing the glycogenolysis.

331

With the object of determining the rôle of asphyxia in the hyperglycemia produced by the drugs which we shall deal with, we thought it necessary first to study the effect of simple asphyxia, without drug action. Carbon monoxide would seem to serve satisfactorily for this purpose since it has no direct action, but acts only by displacement of oxygen. We consider the alkaline reserve capacity of the blood to be a good measure of the asphyxia produced in cases uncomplicated by respiratory or circulatory disturbances other than asphyxial, since according to all available evidences there is in asphyxia diminished oxidation, and the incomplete oxidation results in the failure of the intermediate acid metabolites to be burned to their normal end-products. This accounts for the fall in the alkaline reserve of the blood since these acid bodies are considered to be neutralized by sodium bicarbonate, ammonia, and other buffers as they are formed. The alkaline reserve capacity then is diminished, and should portray the extent of the suboxidation caused by asphyxia.

The carbon monoxide was made by adding concentrated sulfuric acid to oxalic acid and heating. The gas was freed from carbon dioxide by passing through 10 per cent sodium hydroxide. Rabbits were put in a bell jar and asphyxiated by passing in carbon monoxide. The blood sugar was determined by the method of Benedict (6). The alkaline reserve capacity was determined by the method previously described (7). A marked fall in reserve capacity with a mild or slight increase in sugar was obtained (Table I).

Cyanides are considered to produce another type of asphyxia by lessened oxidation in the presence of an abundance of oxygen. Geppert (8) first asserted that hydrocyanic acid depressed physiological oxidations, and has called this "internal asphyxia," since the oxygen is in the ordinary combination with hemoglobin; but there is an inability on the part of the cell to utilize the oxygen. Hyman (9) has shown that the effect in sponges is a primary increase followed by diminution, high concentrations producing practically pure depression. Schoenbein (10) demonstrated that cyanides inhibit the activity of the oxidizing enzymes. Zillessen (11) reported that there was an increase in sugar and lactic acid in the blood under cyanide.

If cyanides depress intracellular oxidation a study of the relation of its glycogenolytic activity to its production of acidosis should be important in connection with the relation of asphyxia and drug hyperglycemia. Accordingly, we injected sodium

TABLE I.

Effect of Carbon Monoxide Asphyxia on Alkaline Reserve Capacity and Blood Sugar Concentration in the Rabbit.

Animal No.	Time.	Alka-line reserve capac-ity.	Blood sugar per cc.	Blood sugar rise.	Remarks.
		cc.	mg.	mg.	
143	8.30 a.m.	0.79	1.25		
	9.16 "				Administered CO for 16 min.
	9.38 "	0.57	1.79	0.54	
	10.02 "				" " " 13 "
	10.38 "	0.53	1.79	0.54	
	11.43 "				" " " 12 "
	12.05 p.m.	0.52	1.74		
	3.00 "				" " " 30 "
	3.35 "	0.37	1.90	0.65	
145	9.30 a.m.	0.68	1.30		
	9.45 "				Administered CO for 27 min.
	10.20 "	0.42	2.43	1.08	
	10.30 "				Killed by CO administered for 19 min.
	10.50 "	0.39	3.26	1.91	
146	4.02 p.m.	0.70	1.35		
	4.12 "				Administered CO for 33 min.
	4.48 "	0.42	1.77	0.42	
	5.25 "	0.54	1.77	0.42	
147	10.15 a.m.	0.80	1.37		
	10.45 "				Administered CO for 35 min.
	11.25 "	0.32	2.00	0.63	
	11.50 "	0.35	2.00	0.63	

cyanide in the ear veins of rabbits and found a marked fall in the reserve capacity of blood with a relatively small rise in blood sugar concentration (Table II).

The large fall in reserve capacity and small rise in sugar concentration were all out of proportion to the large rise of sugar

TABLE II.

Effect of Intravenous Injection of Sodium Cyanide on Alkaline Reserve and Blood Sugar.*

Animal No.	Time.	Alkaline reserve capacity.	Blood sugar per cc.	Blood sugar rise.	Remarks.
		cc.	mg.	mg.	
149	8.00 a.m.		1.20		
	8.15 "				Injected 10 cc. 0.01 м NaCN into ear vein. Time 30 min.
	8.46 "		1.36	0 16	
150	3.40 p.m.		1.15		
	3.45 "				Injected 10 cc. 0.01 м NaCN. Time 30 min. Four convulsions.
	4.15 "		2.30	1.15	
151	4.00 "	0.60	1 36		
	4.30 "				Injected 10 cc. 0.01 м NaCN. Time 30 min. Three convulsions.
	5.15 "	0.28	2.30	0.94	
152	2.15 "	0.60	1.16		
	3.00 "				Injected 7.5 cc. 0.01 м NaCN. Time 30 min. Four mild convulsions.
	3.45 "	0.34	1.66	0.50	
153	10.00 a.m.	0.76	1.33		Double splanchnotomized rabbit.
	10.30 "				Injected 9 cc. 0.01 м NaCN. Time 30 min. Four convulsions.
	11.30 "	0.27	2.20	0.87	
138	11.30 "				2 gm. urethane by stomach tube.
	12.15 p.m.				Started artificial respiration with tracheotomy.
	12.55 "	0.70			
	1.15 "	0.63			
	1.30 "	0.63			
	1.39 "				Injected 0.01 м NaCN for 30 min.
	2.19 "	0.51			" 0.01 м " " 30 "
	2.30 "				
	2.50 "	0.49			
	3.05 "				" 0.01 м " " 30 "
	3.30 "	0.45			

* Folin-Wu method of blood sugar analysis.

and small fall of reserve seen with epinephrine (compare Tables II and XIII). The fall in reserve with cyanide was greater than with carbon monoxide; but the sugar changes were of about the same order of magnitude (compare Tables I and II).

Hydrocyanic acid is one of the strongest stimulants of the respiratory center. Boehm (12) first demonstrated that the respiratory effects were central. The fall in reserve capacity seen with cyanide then might be thought to be due to reduced tension of carbon dioxide in the alveoli and blood because of the increased pulmonary ventilation removing the carbon dioxide from the blood and lungs more promptly than usual. In order to determine whether the fall in reserve observed with cyanide was due to respiratory stimulation, animals were urethanized and given artificial respiration through a tracheal cannula, and then the effect of cyanide was determined. Under these conditions, cyanide still produced a fall in reserve although the artificial respiration was maintained at a constant rate sufficient to maintain quiescence of the respiratory center. See Table II, No. 138. This indicates that the fall in reserve observed with cyanide is not likely due to respiratory center stimulation, since, if it were, the reserve should not have decreased.

Grove and Loevenhart (13), investigating the activity of iodoxybenzoic acid, found that it caused a brief arrest of respiration by depression of the respiratory center presumably because of the active oxygen; and that a definite antagonism exists between hydrocyanic acid and sodium iodoxybenzoate in regard to the respiratory center. Since hydrocyanic acid depresses oxidation, they assume that the antagonism between cyanides and sodium iodoxybenzoate indicates that the latter is capable of accelerating physiological oxidation, because of the effect on the respiration of the one is neutralized by the other.

If sodium iodoxybenzoate acts by increasing intracellular oxidation and cyanides act by decreasing intracellular oxidation, we should expect to be able to see a manifestation of this antagonism on the glycogenolytic mechanism and on the asphyxia of cyanide as portrayed in the alkaline reserve capacity. On injecting iodoxybenzoate in the ear vein of rabbits, we confirmed Loevenhart's findings in regard to the respiratory center; that is, it produced an apnea. Sodium iodoxybenzoate given at such a rate as to

maintain a depressed state of the respiratory center produced a slight hyperglycemia with a rise of the alkaline reserve capacity (Table III).

Since iodoxybenzoate caused a rise in the alkaline reserve capacity, it was necessary to determine whether the rise could

TABLE III.

Effect of Sodium Iodoxybenzoate on Alkaline Reserve and Blood Sugar.

Animal No.	Time.	Alkaline reserve capacity.	Blood sugar per cc.	Blood sugar rise.	Remarks.
		cc.	mg.	mg.	
129	10.30 a.m.	0.69	1.68		
	10.49 "				Injected n/60 Na iodoxybenzoate. Time 47 min.
	11.40 "	0.75	2.04	0.36	
	12.05 p.m.	0.65			
	1.05 "	0.67	1.68		
	1.40 "				Injected n/60 Na iodoxybenzoate. Time 30 min.
	2.14 "	0.69	1.92	0.24	
130	4.00 "	0.75	1.34		
	4.30 "				Injected 8 cc. n/90 Na iodoxybenzoate. Time 30 min.
	5.14 "	0.83	1.78	0.44	
131	11.30 a.m.	0.73	1.32		
	11.40 "				Injected 5.5 cc. n/90 Na iodoxybenzoate. Time 30 min.
	12.12 p.m.	0.86	1.70	0.38	
	12.32 "	0.83	1.62	0.30	
162	2.30 "	0.75	1.32		
	2.47 "				Injected 8 cc. n/90 Na iodoxybenzoate. Time 30 min.
	3.19 "	0.78	1.67	0.35	

not be accounted for by retention of carbon dioxide due to depression of the respiratory center. We therefore injected sodium iodoxybenzoate into the ear veins of artificially respirated, urethanized rabbits and found that the reserve did not increase. Hence, we conclude that iodoxybenzoate produces a rise in re-

serve capacity by depression of the respiratory center causing a retention of carbon dioxide in the blood (Table IV).

Table IV also shows the effect on the alkaline reserve capacity of subcutaneous injection of epinephrine in artificially respirated animals. Epinephrine produced a fall of reserve. This indicates that the fall of reserve cannot be due to respiratory stimulation, and confirms previous work in this laboratory (5).

For completeness, we tried iodoxybenzoate in double splanchnotomized and left adrenalectomized-right splanchnotomized animals and found that it no longer produced hyperglycemia but did produce a rise in the alkaline reserve capacity. The glycogenolytic action of iodoxybenzoate in normal animals then is central, probably by stimulation of the central glycotaxic mechanism. However, if an overdose of iodoxybenzoate is given, so as to maintain apnea, an increase in the blood sugar concentration and a fall in the alkaline reserve capacity is obtained (see Table V). This can be explained on the basis of the apnea resulting in a lack of oxygen. On the other hand in cases of small doses of iodoxybenzoate the depression of the respiratory center was sufficient to produce some accumulation of carbon dioxide without at the same time producing actual oxygen want.

To determine whether the antagonism seen between iodoxybenzoate and cyanide on the respiratory center could also be seen in regard to cyanide hyperglycemia and reserve fall, we injected into an ear vein of a rabbit a single solution containing cyanide and iodoxybenzoate, which, tested electrometrically, gave no evidence of chemical interaction. By trial a mixture was obtained which because of the predominance of iodoxybenzoate, more than neutralized the effect of cyanide so that the respiration was depressed. We found that cyanide still produced the large fall in reserve capacity, the sugar change, and typical cyanide convulsions even when the effect of cyanide on the respiratory center was entirely overbalanced by the depression produced by iodoxybenzoate (see Table VI).

Hence iodoxybenzoate is not a good antidote to the effects observed with cyanide, other than control of the respiratory center and possibly other medullary centers. Grove and Loevenhart consider that iodoxybenzoate causes depression of the respiratory center by increasing intracellular oxidation. Our evidence

TABLE IV.

Effect of Sodium Iodoxybenzoate and Epinephrine on Alkaline Reserve
Capacity of Whole Blood in Artificially Respirated Rabbits.

Animal No.	Time.	Alkaline reserve capacity.	Remarks.
		cc.	
135	10.19 a.m.		1 gm. urethane per kilo.
	10.45 "		Started artificial respiration with tracheotomy.
	10.50 "	0.69	
	11.15 "	0.65	
	12.00 m.	0.56	
	12.30 p.m.	0.58	
	12.45 "		Injected 5 cc. N 30 Na iodoxybenzoate into ear vein. Time 20 min.
	1.06 "	0.57	
136	9.30 a.m.		1 gm. urethane per kilo.
	10.20 "		Started artificial respiration.
	10.50 "	0.65	
	11.10 "	0.66	
	11.30 "	0.62	
	11.41 "		Injected 5 cc. N 90 Na iodoxybenzoate. Time 55 min.
	12.39 p.m.	0.62	
	12.45 "		Injected 5 cc. N 90 Na iodoxybenzoate. Time 30 min.
	1.20 "	0.60	
	1.30 "		Injected 0.5 cc. adrenalin subcutaneously.
	2.30 "	0.55	
137	3.00 "		1 gm. urethane per kilo.
	4.00 "		Started artificial respiration.
	4.15 "	0.66	
	4.30 "	0.65	
	5.00 "	0.63	
	5.05 "		Injected 5 cc. N 90 iodoxybenzoate. Time 35 min.
	5.45 "	0.63	
	6.00 "		Injected 5 cc. N 90 iodoxybenzoate. Time 30 min.
	6.34 "	0.61	
139	10.35 a.m.		1 gm. urethane per kilo.
	11.25 "		Started artificial respiration.
	12.00 m.	0.61	
	12.15 p.m.	0.62	
	12.30 "	0.61	
	12.45 "		Injected 0.5 cc. adrenalin subcutaneously.
	12.50 "		Injected N 90 iodoxybenzoate. Time 35 min.
	1.30 "	0.54	

is not directly confirmatory to this view, although it may not be antagonistic since there is a possibility of a difference of general visceral susceptibility in response to iodoxybenzoate compared

TABLE V.

Effect of Sodium Iodoxybenzoate on Alkaline Reserve and Blood Sugar Concentration of Double Splanchnotomized Rabbits.

Animal No.	Time.	Alkaline reserve capacity.	Blood sugar per cc.	Blood sugar rise.	Remarks.
		cc.	mg.	mg.	
163	10.00 a.m.	0.76	1.59		
	10.35 "				Injected N 90 Na iodoxybenzoate. Time 36 min. Respiration was depressed; but there was at no time apnea.
	11.15 "	0.81	1.63	0.04	
161	10.19 "	0.76	1.32		
	10.40 "				Injected 20 cc. N/90 Na iodoxybenzoate. Time 30 min. Complete cessation of respiration several times during period of injection.
	11.15 "	0.46	2.03	0.71	.
157	8.00 "		1.17		
	8.30 "				Injected 18 cc. N 90 Na iodoxybenzoate. Time 40 min. Complete cessation of respiration several times during period of injection.
	9.15 "		1.71	0.54	
156	4.00 p.m.		1.29		
	4.25 "				Injected as in Animal 157.
	5.00 "		1.90	0.61	

to the medullary center responses as shown by Loevenhart and Eyster (14) to be true in the case of the heart.

The question of ether hyperglycemia has been extensively studied with the investigators being unable to reach an agreement in opinion. Keeton and Ross (15) claim that ether hypergly-

cemia is abolished after double splanchnotomy in dogs; while
Stewart and Rogoff (16) claim that it is obtained after right
adrenalectomy and left splanchnotomy in cats, and after double
adrenalectomy in rabbits. Fujii (17) found hyperglycemia in
rabbits after double splanchnotomy, but less than in the normal
controls. We used ether anesthesia in double splanchnotomized
and left adrenalectomized-right splanchnotomized rabbits by

TABLE VI.

*Effect of Injection of Sodium Cyanide and Sodium Iodoxybenzoate on Alkaline
Reserve Capacity and Blood Sugar.*

Animal No.	Time.	Alkaline reserve capacity.	Blood sugar per cc.	Blood sugar rise.	Remarks.
		cc.	*mg.*	*mg.*	
140	8.30 a.m.	0.75	1.41		Injected mixture of NaCN and Na iodoxybenzoate into ear vein. Time 30 min. Kept respiration at normal rate.
	9.00 "				
	9.45 "	0.32	2.36	0.95	
141	1.00 p.m.	0.70	1.40		Injected solution as in No. 140.
	1.30 "				
	2.05 "	0.24	2.29	0.89	
142	3.05 "	0.71	1.35		Injected mixture of cyanide and iodoxybenzoate with predominance of iodoxybenzoate effect on the respiratory center. Some cyanide convulsions were observed.
	3.30 "				
	4.10 "	0.30	1.95	0.60	

the ordinary towel method of administration of ether to determine
whether the ether action is central or peripheral. The advantage
in cutting the splanchnics on one side and extirpating the adrenal
on the other as pointed out by Stewart and Rogoff is that the
innervation of the liver is not seriously interfered with as in
double splanchnotomy, and at the same time the effect of adrenal
intermediation is eliminated, since the remaining gland is de-
nervated. The evidence obtained indicates that ether is gly-

cogenolytic in such animals essentially like unoperated animals, and hence that the action is peripheral, confirming Stewart and Rogoff. See Tables VII and VIII.

Since with the use of the towel method, there is the possibility of asphyxia due to lack of oxygen supply to the lungs, we introduced a catheter into the trachea and then gave ether by continuous insufflation. The results were essentially the same in operated and unoperated animals. See Tables IX and X.

This shows that ether hyperglycemia is not essentially central, but is predominantly a peripheral action. The same operated animals did not respond to quinine nor to picrotoxin but did to epinephrine. The failure to respond to quinine and picrotoxin shows that the operations were successful for the elimination of central stimulation of the glycotaxic center, since quinine (18) and picrotoxin (19) produce hyperglycemia by central stimulation. The normal response to epinephrine shows that the liver is not deficient in glycogen.

Acidosis has commonly been considered the cause of ether hyperglycemia. Therefore we suppose that if this is true, a preexisting acidosis should cause the ether glycogenolysis to be much more marked, because here we have depletion of the alkaline reserve materials within the cells to start with since the reserve of the cell would be drawn on in cases of diminished capillary blood reserve and hence there would be far less resistance to the production of acidosis by ether. We find no significant synergism, but summation or slightly higher sugar changes than in normal controls (see Table XI). Loevenhart (20) considers that acids depress intracellular oxidation and that alkalies increase intracellular oxidation. The slightly higher sugar changes than in controls without acids might be considered to be due to the decreased intracellular oxidation produced by acids plus that of ether.

Ether itself produces both a fall in alkaline reserve capacity and a rise in blood sugar. We found it impossible to obtain a comparable sugar increase by acid administration even though the reserve capacity was brought down to a far lower value than it falls with ether (see Table XI). It may be argued that the alkaline reserve capacity does not represent a true picture of what is going on in the liver, and that ether acidosis is primarily

TABLE VII.

*Effect of Ether Anesthesia on Blood Sugar Concentration of Normal Rabbits.
Ether Administered by the Ordinary Towel Method.*

| Animal No. | Blood sugar per cc. | | Blood sugar rise. | Remarks. In each experiment the animals were anesthetized for 1 hr. |
	Before.	After.		
	mg.	*mg.*	*mg.*	
1	1.66	2.00	0.34	
2	1.30	1.85	0.55	
4	1.70	1.92	0.22	
5	0.90	1.55	0.65	
6	1.21	1.48	0.27	
9	1.30	1.60	0.30	
27	1.40	3.36	1.96	Deep etherization.
29	1.33	3.15	1.82	" "
30	1.26	2.30	1.04	
31	1.33	1.56	0.23	

TABLE VIII.

*Effect of Ether Anesthesia on Blood Sugar Concentration of Double Splanch-
notomized Rabbits. Ether Administered on Towel.*

| Animal No. | Blood sugar per cc. | | Blood sugar rise. | Remarks. Anesthesia maintained for 1 hr. Light etherization. |
	Before.	After.		
	mg.	*mg.*	*mg.*	
10	1.32	1.68	0.36	
11	1.44	1.95	0.51	
12	1.23	1.69	0.46	
13	1.23	2.00	0.77	
14	1.63	1.90	0.27	
16	1.21	1.48	0.27	
17	1.27	1.68	0.41	

Effect of ether on blood sugar of rabbits with left adrenalectomy and
right splanchnotomy.

18	1.27	2.85	1.58	
19	1.14	1.79	0.65	
20	1.31	2.04	0.73	
21	1.43	3.12	1.69	
22	1.34	1.57	0.23	

TABLE IX.

Effect of Ether Anesthesia by Continuous Insufflation on Blood Sugar and Alkaline Reserve Capacity of Whole Blood. Normal Rabbits.

Animal No.	Time.	Alkaline reserve capacity.	Blood sugar per cc.	Blood sugar rise.	Remarks.
		cc.	*mg.*	*mg.*	
48			1.37		
	1 hr. ether.		1.78	0.41	
52			1.42		
	1 hr. ether.		3.57	2.15	Deep etherization.
53			1.48		
	1 hr. ether.		3.40	1.92	Deep etherization.
55			1.37		
	1 hr. ether.		3.16	1.79	Deep etherization.
56			1.20		
	1 hr. ether.		1.95	0.75	
57			1.41		
	1 hr. ether.		1.69	0.28	
104	9.30 a.m.	0.75	1.31		
	10.05 "				Started ether insufflation.
	11.15 "	0.75	1.71	0.40	
	12.00 m.	0.74	1.91	0.60	
	12.55 p.m.	0.66	2.39	1.08	
105	9.30 a.m.	0.76	1.14		
	10.15 "				Started ether insufflation.
	11.00 "	0.58	3.13	1.99	
	11.50 "	0.56	3.13	1.99	
106	9.00 "	0.76	1.05		
	9.35 "				Started ether insufflation.
	10.35 "	0.71	1.80	0.75	
	11.40 "	0.72	1.80	0.75	
	12.35 p.m.	0.70	1.99	0.94	
108	11.50 a.m.	0.74	1.15		
	12.25 p.m.				Started ether insufflation.
	1.40 "	0.74	2.38	1.23	
	2.55 "	0.74	2.50	1.35	
113	9.15 a.m.	0.68	1.46		
	9.45 "				Started ether insufflation.
	10.50 "	0.61	3.16	1.70	

TABLE X.

Effect of Ether Anesthesia by Continuous Insufflation on Blood Sugar Concentration of Double Splanchnotomized and Left Adrenolectomized— Right Splanchnotomized Rabbits.

Animal No.	Time.	Blood sugar per cc.	Blood sugar rise.	Remarks.
		mg.	*mg.*	
54	8.00 a.m.	1.48		
	8.20 "			Started ether insufflation.
	9.20 "	2.66	1.18	
58	1.00 p.m.	1.27		
	1.30 "			Started ether insufflation.
	2.30 "	1.52	0.25	
59	5.00 "	1.22		
	5.20 "			Started ether insufflation.
	6.20 "	1.74	0.52	
62	2.25 "	1.21		
	2.30 "			Started ether insufflation.
	3.05 "	2.13	0.92	
	3.30 "	2.34	1.13	
	5.35 "	2.44	1.23	
64	4.30 "	1.35		
	4.40 "			Started ether insufflation.
	5.40 "			Stopped " "
	5.50 "	1.95	0.60	
65	9.10 a.m.	1.24		
	9.15 "			Started ether insufflation.
	10.15 "	1.56	0.32	
	10.52 "	1.82	0.58	
68	2.25 p.m.	1.24		
	2.45 "			Started ether insufflation. Very light.
	3.15 "	1.47	0.23	
	3.45 "			Ether turned off. Gave air insufflation.
	3.55 "	1.74	0.50	

hepatic; but the acidosis produced by giving of acid by stomach affects the portal system first also.

If the glycogenolytic action of ether is due to acidosis, alkali administration should diminish the effect of ether as a

glycogenolytic agent. Our results show a suggestive but not very convincing evidence of diminished effect under alkali (see Table XII). The slight diminution of ether effect sometimes

TABLE XI.

Effect of 1 Per Cent HCl on Ether Hyperglycemia and Alkaline Reserve Status. Acid Administered by Stomach Tube in the Rabbit.

Animal No.	HCl	Time for acid.	Reserve before acid.	Reserve before ether.	Reserve after 1 hr. of ether.	Sugar rise by acid.	Sugar rise by acid plus ether.	Remarks.
		a. Light ether.						
		min.	cc.	cc.	cc.		mg. per cc.	
73	70	65					0.81	
87	40	120				0.11	0.38	
94	61	120	0.75	0.65	0.56	0	0.87	
98	60	150	0.76	0.50	0.57	0	0.51	
99	63	100		0.54	0.50		0.47	
102	70	195	0.74	0.58	0.55	0	0.57	
112	90	240	0.70	0.47	0.44	0	0.91	
118	125	160		0.39	0.37	0.89	1.48	
119	120	120	0.67	0.38	0.38	0	0.75	
120	160	195	0.66	0.38	0.31	0.59	1.55	
126	155	90		0.44	0.38		0.38	
126				0.50			0.72	Next day without acid.
		b. Deeper etherization.						
114	135	150		0.37	0.35	0.05	1.39	
110	110	210		0.43	0.39	0.22	1.90	
103	60	180	0.87	0.58	0.58	0	2.81	
97	57	150	0.81	0.56	0.56	0	1.40	
93	63	110	0.74	0.63	0.65	0.10	1.63	
100	59	150	0.78	0.54	0.50	0	1.60	

Continued ether in No. 103 for 3 hrs., with no further fall in alkaline reserve, but with sugar rise of 3.96 over normal.

Where large amounts of acid were given, it was diluted with water and given in three doses at various intervals.

seen under alkali may be regarded as being due to alkalies tending to increase intracellular oxidations. The work with acids and alkalies thus indicates that acidosis is not the cause of ether

hyperglycemia, but that decreased intracellular oxidation may be a factor. Thus acidosis and increased glycogenolysis may be parallel phenomena, and to a certain extent, manifestations of suboxidation.

TABLE XII.

Effect of Alkali on Ether Hyperglycemia and Alkaline Reserve Status. Sodium Bicarbonate Administered by Stomach Tube in the Rabbit.

Animal No.	Sodium bicarbonate.	Time for alkali.	Reserve before alkali.	Reserve before ether.	Reserve after 1 hr. of ether.	Sugar rise after 1 hr. of ether.	Remarks.
	gm.	min.	cc.	cc.	cc.	mg. per cc.	
116	2.75	235	0.57	0.74	0.70	0.43	Very light ether.
117	2.75	260	0.75	0.86	0.77	0.65	" " "
121	3.00	195	0.75	0.85	0.79	0.67	
123	4.00	230	0.81	0.90	0.86.	0.68	

TABLE XIII.

Effect of Epinephrine on Alkaline Reserve Capacity of Whole Blood and Blood Sugar Concentration in the Rabbit. Control Series.

Animal No.	Blood sugar per cc.		Blood sugar rise.	Alkaline reserve capacity.		Remarks. 0.5 cc. adrenalin injected in every experiment. Readings after 1 hr.
	Before.	After 1 hr.		Before.	After.	
	mg.	mg.	mg. per cc.	cc.	cc.	
25	1.33	2.94	1.61			
33	1.41	3.33	1.92			
50	1.43	3.10	1.67			
51	1.62	3.39	1.77			
133	1.40	3.16	1.76			
10*	0.98	2.51	1.53	0.72	0.55	
11*	0.94	2.51	1.57	0.62	0.55	
12*	1.19	2.83	1.64	0.66	0.50	
13*	0.84	1.74	0.90	0.60	0.56	
14*	0.94	2.83	1.89	0.60	0.56	
15*	0.94	2.51	1.57	0.63	0.60	
16*	0.98	2.06	1.08	0.63	0.60	

* Reprinted from previous work (5).

In the work on epinephrine (5), it has been shown that the glycogenolysis produced could not be explained on the basis of hepatic asphyxia or acidosis. If epinephrine and ether both produced glycogenolysis by acidosis we should expect that the

acidosis produced by the one would enable the acidosis produced by the other to show greater effect on glycogenolysis, since the threshold for increased glycogenolytic activity would have already been reached. Therefore, epinephrine and ether simultaneously should show a synergistic effect on the glycogenolysis produced, if they act by a common mechanism. On giving epinephrine and ether simultaneously, we found no demonstrable synergism but summation of glycogenolytic effects (see Tables XIII and

TABLE XIV.

Effect of Simultaneous Administration of Ether and Epinephrine on the Blood Sugar Concentration in the Rabbit.

Animal No.	Blood sugar per cc.		Blood sugar rise.	Remarks. 0 5 cc. adrenalin injected subcutaneously at start of ether anesthesia.
	Before.	After 1 hr.		
	mg.	*mg.*	*mg. per cc.*	
23	1.60	5.00	3.40	
24	1.54	4.21	2.67	
26	1.47	3.28	1.81	
32	1.49	2.96	1.47	
34	1.29	3.30	2.19	
35	1.36	2.87	1.51	
36	1.60	5.55	3.95	Very deep etherization.
37	1.55	5.10	3.55	" " "
38	1.33	3.92	2.59	
39	1.38	3.33	1.95	
40	1.29	4.27	2.98	
41	1.55	2.56	1.01	
42	1.38	3.44	2.06	
43	1.45	3.08	1.63	
44	1.60	3.40	1.80	
45	1.37	2.58	1.21	
46	1.21	2.82	1.61	
47	1.48	3.80	2.42	

XIV). This indicates that the ether and epinephrine each produced their respective hyperglycemias by independent actions.

With the idea of possibly throwing more light on the mechanism of epinephrine hyperglycemia, we tried iodoxybenzoate injection during the period of epinephrine action and found that the alkaline reserve capacity of the blood may be kept up or even elevated, but the glycogenolysis produced by epinephrine was unchanged (see Table XV).

TABLE XV.

Effect of Sodium Iodoxybenzoate on Epinephrine Hyperglycemia and Alkaline Reserve Status in the Rabbit.

Animal No.	Time.	Alkaline reserve capacity.	Blood sugar per cc.	Blood sugar rise.	Remarks.
		cc.	*mg.*	*m.l. per cc.*	
128	10 20 a.m.	0.70	1.13		
	10.42 "				Injected 0.5 cc. adrenalin subcutaneously.
	10.44 "				Injected 10 cc. N/30 Na iodoxybenzoate into ear vein. Time 46 min.
	11.34 "	0.68	3.10	1.97	Respiration not completely controlled.
132	3.30 p.m.	0.70	1.49		
	4 05 "				Injected 0.5 cc. adrenalin.
	4.06 "				Injected N/90 iodoxybenzoate. Time 44 min. Intermittent apnea and hyperpnea.
	4.45 "	0.65	2 85	1.36	
134	4.10 "	0.72	1.41		
	4 22 "				Injected 0.5 cc. adrenalin. Injected 10 cc. N/90 iodoxybenzoate.
	5.04 "	0.78	2 72	1.31	
	5.44 "	0.64	3.41	2 00	

GENERAL INTERPRETATION.

A summary comparison of the actions of ether, epinephrine, carbon monoxide, and cyanides on the blood sugar concentration and alkaline reserve capacity seems to be profitable. We have demonstrated that cyanide and carbon monoxide (in other words asphyxia) produce a much greater fall in reserve and much less sugar rise than epinephrine or ether. Since the fall in reserve is presumably caused by diminished oxidation, the hyperglycemia of ether, epinephrine, carbon monoxide, and cyanide cannot in each case be caused by diminished intracellular oxidation, if this is adequately measured by the alkaline reserve capacity. The

fall in reserve should be a measure of the same diminution of oxidation and this same diminution of oxidation should lead to a relatively definite glycogenolysis, providing the circulatory and respiratory factors are not sufficiently altered as to vitiate our interpretation.

If this reasoning is logical, it then follows that the hyperglycemia produced by ether, cannot all be accounted for on the basis of asphyxia or acidosis. In regard to epinephrine there is absolutely no available evidence of any contributary action of asphyxia in hyperglycemia production by this drug.

BIBLIOGRAPHY.

1. Stewart. G. N., Manual of physiology, New York, 8th edition, 1918, 548.
2. Sansum, W. M., and Woodyatt, R. T., *J. Biol. Chem.*, 1915, xxi, 1.
3. Ross, E. L., and McGuigan, H., *J. Biol. Chem.*, 1915, xxii, 407.
4. Ritzmann, H., *Arch. exp. Path. u. Pharmakol.*, 1909, lxi, 231.
5. Tatum, A. L., *J. Pharmacol. and Exp. Therap.*, 1921, xvii, 395.
6. Benedict, S. R., *J. Biol. Chem.*, 1918, xxxiv, 203.
7. Tatum, A. L., *J. Biol. Chem.*, 1920, xli, 59.
8. Geppert, J., *Z. klin. Med.*, 1889, xv, 208.
9. Hyman, L. H., *Am. J. Physiol.*, 1916, xl, 238.
10. Schoenbein, C. F., *Z. Biol.*, 1867, iii, 140.
11. Zillessen, H., *Z. physiol. Chem.*, 1891, xv, 387.
12. Boehm, R., *Arch. exp. Path. u. Pharmakol.*, 1874, ii, 129.
13. Grove, W. E., and Loevenhart, A. S., *J. Pharmacol. and Exp. Therap.*, 1911–12, iii, 101.
14. Loevenhart, A. S., and Eyster, J. A. E., *J. Pharmacol. and Exp. Therap.*, 1913–14, v, 21.
15. Keeton, R. W., and Ross, E. L., *Am. J. Physiol.*, 1919, xlviii, 146.
16. Stewart, G. N., and Rogoff, J. M., *Am. J. Physiol.*, 1917, xliv, 543; 1918, xlvi, 90.
17. Fujii, I., *Tohoku J. Exp. Med.*, 1921, ii. 170.
18. Tatum, A. L., and Cutting, R. A., *J. Pharmacol. and Exp. Therap.*, 1922, in press.
19. Tatum, A. L., *J. Pharmacol. and Exp. Therap.*, 1922, in press.
20. Loevenhart, A. S., *Arch. ges. Physiol.*, 1913, cl, 379.

ON OXIDATION OF TERTIARY HYDROCARBONS.

By P. A. LEVENE and F. A. TAYLOR.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

Hydrocarbons with a tertiary carbon atom have attracted little attention. Few have been prepared synthetically and of those occurring in nature only a few have been investigated from the view-point of their structures. The lack of interest in this group of substances may be due to the belief in their inertness. However, there is accumulating evidence to show that the lack of reactivity of the aliphatic hydrocarbons is not as great as accepted in the past, particularly in the case of the hydrocarbons with a tertiary carbon atom. The fact that 3-methyl-pentane is capable of decolorizing a solution of potassium permanganate has been known since 1901.[1] The products of oxidation of this substance were not analyzed.

A considerable number of naturally occurring substances, among which are the higher fatty acids, are derivatives of tertiary hydrocarbons. The knowledge of their structures depends on that of the structures of the hydrocarbons.

The present work was undertaken in 1918 in cooperation with L. H. Cretcher. The work was interrupted due to the conditions of war and at that time a short publication on the subject was published in this Journal.[2] Subsequently, the work was continued by the present writers.

Recently, the subject of oxidation of saturated hydrocarbons attracted considerable attention. However, on the special problem of oxidation of tertiary hydrocarbons no additional work has been reported.

[1] Zelinsky, N., and Zelikow, J., *Ber. chem. Ges.*, 1901, xxxiv, 2865.
[2] Levene, P. A., and Cretcher, L. H., Jr., *J. Biol. Chem.*, 1918, xxxiii, 505.

The present communication contains a report on the synthesis of five tertiary hydrocarbons. Their properties are tabulated below. The method of preparation was the same as employed in the previous work; namely, through the malonic ester synthesis by the following series of reactions:

$$
\begin{array}{c}
R_1 \\ \diagdown \\ C \\ \diagup \\ R_2
\end{array}
\begin{array}{c}
COOR \\ \\ COOR
\end{array}
\rightarrow
\begin{array}{c}
R_1 \\ \diagdown \\ C \\ \diagup \\ R_2
\end{array}
\begin{array}{c}
COOH \\ \\ COOH
\end{array}
\rightarrow
\begin{array}{c}
R_1 \\ \diagdown \\ CH \cdot COOH \\ \diagup \\ R_2
\end{array}
\rightarrow
\begin{array}{c}
R_1 \\ \diagdown \\ CH - COOR \\ \diagup \\ R_2
\end{array}
\rightarrow
$$

$$
\begin{array}{c}
R_1 \\ \diagdown \\ CHCH_2OH \\ \diagup \\ R_2
\end{array}
\rightarrow
\begin{array}{c}
R_1 \\ \diagdown \\ CHCH_2I \\ \diagup \\ R
\end{array}
\rightarrow
\begin{array}{c}
R_1 \\ \diagdown \\ CHCH_3 \\ \diagup \\ R_2
\end{array}
$$

This tedious method of preparation was adopted because paraffins are freed from impurities with great difficulty and therefore can be obtained in a sufficient degree of purity best by a set of reactions each simple in its nature and when each intermediary substance can be isolated and purified.

Turning to the oxidation by means of permanganate solution it was found that for 5-methyl-nonane the conditions employed by Levene and Cretcher were the most favorable. The higher tertiary hydrocarbons, however, were not odixized under identical conditions. The failure may be due to the lowering in solubility of the hydrocarbons with increase of their molecular weights. The conditions for the oxidation of substances with higher molecular weights will have to be worked out.

Among the acids formed on oxidation of 5-methyl-nonane, two were identified; namely, acetic and butyric acids. The presence of some formic acid was detected. On the basis of this, the oxidation seems to progress in the main as follows:

$$
\overset{\displaystyle CH_3}{CH_3(CH_2)_3\,CH} - (CH_2)_3\,CH_3 \rightarrow CH_3(CH_2)_3\,\overset{\displaystyle CH_3}{CO} + CH_3(CH_2)_2\,COOH \rightarrow
$$
$$
CH_3(CH_2)_3\,CO - CH_3 \rightarrow CH_3(CH_2)_2\,COOH + CH_3\,COOH
$$

General conclusions as to the progress of oxidation of tertiary hydrocarbons will be reached only after the observations have been extended to a greater number of these substances.

Hydrocarbons.

No.	Hydrocarbon.	Boiling point.		D_4^{20}	N_D^{20}	M_D^{20}	
		Temperature.	Pressure.			Calculated.	Found.
		°C.	mm.				
1	3-Methyl-heptane.	120–122	755	0.7069	1.3980	39.14	38.92
2	3-Methyl-nonane.	165.5–166.5	751	0.7354	1.4126	48.38	48.12
3	5-Methyl-nonane.	164–166	755	0.7319	1.4116	48.38	48.24
4	5-Propyl-nonane.	204–205	763	0.7559	1.4228	57.62	57.25
5	5-Methyl-dodecane.	225.5–227	758	0.7576	1.4244	62.23	62.02

EXPERIMENTAL.

All melting and boiling point temperatures recorded in the following experiments were determined with standardized Anschütz thermometers.

Diethyl Ethyl-Malonate.—Ethyl iodide was condensed with malonic ester in the presence of sodium ethylate as described by Conrad.[3] The yield of substituted ester was 83 per cent of the theoretical. It boiled at 206–208°C.

Diethyl Butyl-Ethyl-Malonate.—This ester was prepared by the condensation of butyl iodide and diethyl ethyl-malonate. Sodium (12.2 gm.) was dissolved in absolute alcohol (150 cc.) and the diethyl ethyl-malonate added. Butyl iodide (112 gm.) was then cautiously introduced and the solution boiled on the water bath over night. After the solution had become neutral, most of the alcohol was removed by distillation. The residue was taken up in ether, filtered to remove the sodium iodide, and distilled. The ester boiled at 128–129°C. at 7 mm. The yield was 62 per cent of the theoretical. Although prepared earlier by Raper[4] the ester had not been characterized.

$$D_4^{20} = 0.9646 \qquad N_D^{20} = 1.4284$$

0.0990 gm. substance: 0.2312 gm. CO_2 and 0.0874 gm. H_2O.

$C_{13}H_{24}O_4$. Calculated. C 63.93, H 9.84, M_D^{20} 65.54.

Found. " 63.68, " 9.88, " 65.13.

---

[3] Conrad, M., *Ann. Chem.*, 1880, cciv, 134.

[4] Raper, H. S., *J. Chem. Soc.*, 1907, xci, 1837.

Butyl-Ethyl-Malonic Acid.—For the preparation of the acid, the above ester was not isolated but was saponified by heating the solution in which it was formed with a large excess of sodium hydroxide. After crystallization from high boiling gasoline, the acid melted at 115°C.

0.1020 gm. substance: 0.2168 gm. CO_2 and 0.0798 gm. H_2O.
$C_9H_{16}O_4$. Calculated. C 57.44, H 8.51.
Found.   " 57.96, " 8.74.

2-Ethyl-Hexylic Acid.—Butyl-ethyl-malonic acid was heated at 180°C. as long as carbon dioxide was evolved and the residue distilled. It boiled at 228–229°C. at 755 mm. Raper[4] found a boiling point of 225°C.

0.1020 gm. substance: 0.2500 gm. CO_2 and 0.1028 gm. H_2O.
$C_8H_{16}O_2$. Calculated. C 66.66, H 11.11.
Found.   " 66.83, " 11.28.

Ethyl 2-Ethyl-Hexylate.—The acid was esterified in the usual manner with absolute alcohol and sulfuric acid. The yield was 91 per cent of the theoretical. The ester boiled at 189–191°C. at 766 mm.

$$D_4^{20} = 0.8628 \qquad N_D^{20} = 1.4128$$
0.1006 gm. substance: 0.2566 gm. CO_2 and 0.1040 gm. H_2O.
$C_{10}H_{20}O_2$. Calculated. C 69.76, H 11.62, M_D^{20} 50.03.
Found.   " 69.56, " 11.56, " 49.69.

2-Ethyl-Hexanol.—Ethyl 2-ethyl-hexylate was reduced by means of sodium and absolute alcohol. The method has been described by Levene and Cretcher.[2] The yield of alcohol was 78 per cent of the theoretical and in addition to this 10 per cent of the ester was recovered as 2-ethyl-hexylic acid. The alcohol boiled at 181–183°C. at 743 mm.

$$D_4^{20} = 0.8328 \qquad N_D^{20} = 1.4328$$
0.1016 gm. substance: 0.2752 gm. CO_2 and 0.1070 gm. H_2O.
$C_8H_{18}O$. Calculated. C 73.85, H 13.85, M_D^{20} 40.67.
Found.   " 73.86, " 13.77, " 40.55.

2-Ethyl-Hexyl Iodide.—This iodide was prepared by heating the alcohol (50 gm.) with 1.1 equivalents of iodine and an excess

of red phosphorus at 180°C. for 1 hour. The product was washed, dried, and distilled. The yield was 80 per cent of the theoretical. The iodide boiled at 89–90°C. at 11 mm.

$$D_4^{20} = 1.3365$$

0.2234 gm. substance: 0.2170 gm. Ag I (Carius).
$C_8H_{17}I.$ Calculated. I 52.92.
                    Found.   " 52.48.

3-Methyl-Heptane.—The iodide was reduced in glacial acetic acid with zinc dust. The solution was saturated with hydrogen chloride and heated on the water bath for 3 days with frequent introduction of small portions of zinc. The hydrocarbon was then distilled in a current of steam, collected in ether, and distilled with a fractionating column. It boiled at 120–122°C. at 755 mm. Clarke[5] found 117.6°C. The yield was 84 per cent of the theoretical.

$$D_4^{20} = 0.7069 \qquad\qquad N_D^{20} = 1.3980$$

0.0965 gm. substance: 0.2994 gm. CO_2 and 0.1356 gm. H_2O.
$C_8H_{18}.$ Calculated. C 84.21, H 15.79, M_D^{20} 39.14.
                    Found.   " 84.59, " 15.72, " 38.92.

Diethyl 1-Methyl-Heptyl-Malonate.—1-Methyl-heptyl iodide was condensed with malonic ester (1.25 equivalents) in the usual manner. The yield was 80 per cent of the theoretical. The ester boiled at 157–158°C. at 10 mm.

$$D_4^{20} = 0.9496 \qquad\qquad N_D^{20} = 1.4324$$

0.1016 gm. substance: 0.2472 gm. CO_2 and 0.0928 gm. H_2O.
$C_{15}H_{28}O_4.$ Calculated. C 66.18, H 10.29, M_D^{20} 74.78.
                    Found.   " 66.34, " 10.22, " 74.36.

1-Methyl-Heptyl-Malonic Acid.—The ester was saponified with a large excess of sodium hydroxide. The acid was liberated from the sodium salt with dilute hydrochloric acid. Since it could not be made to crystallize, it was collected in ether, dried, and the ether removed by distillation. The residue was converted into the corresponding substituted acetic acid.

[5] Clarke, L., *J. Am. Chem. Soc.*, 1911, xxxiii, 521.

3-Methyl-Nonylic Acid.— Crude 1-methyl-heptyl-malonic acid was heated at 180°C. as long as carbon dioxide was evolved. The residue was then distilled. It boiled at 147–148°C. at 12 mm.

$$D_4^{20} = 0.9012 \qquad\qquad N_D^{20} = 1.4342$$

0.0998 gm. substance: 0.2546 gm. CO_2 and 0.1036 gm. H_2O.

$C_{10}H_{20}O_2$. Calculated. C 69.77, H 11.63, M_D^{20} 49.92.

Found.   " 69.57, " 11.61, " 49.72.

Ethyl 3-Methyl-Nonylate.—The acid was esterified in the usual manner with alcohol and sulfuric acid. It boiled at 115°C. at 13 mm.

$$D_4^{20} = 0.8653 \qquad\qquad N_D^{20} = 1.4240.$$

0.0982 gm. substance: 0.2598 gm. CO_2 and 0.1080 gm. H_2O.

$C_{12}H_{24}O_2$. Calculated. C 72.00, H 12.00, M_D^{20} 59.27.

Found.   " 72.15, " 12.19, " 58.98.

3-Methyl-Nonanol.— This alcohol was obtained by the reduction with sodium and alcohol of the corresponding ester. The yield was over 90 per cent. It boiled at 103–103.5°C. at 9 mm.

$$D_4^{20} = 0.8342 \qquad\qquad N_D^{20} = 1.4361$$

0.1014 gm. substance: 0.2826 gm. CO_2 and 0.1296 gm. H_2O.

$C_{10}H_{22}O$. Calculated. C 75.94, H 13.92, M_D^{20} 49.91.

Found.   " 76.00, " 14.29, " 49.53.

3-Methyl-Nonyl Iodide.—3-Methyl-nonanol was heated for 1 hour at 180°C. with 1.1 equivalents of iodine and an excess of red phosphorus. The iodide was then washed, dried, and distilled. It boiled at 115°C. at 10 mm. The yield was 90 per cent of the theoretical.

$$D_4^{20} = 1.2515$$

0.2430 gm. substance: 0.2430 gm. AgI (Carius).

$C_{10}H_{21}I$. Calculated. I 47.39.

Found.   " 47.36.

3-Methyl-Nonane.—This hydrocarbon was prepared in the same manner as the 3-methyl-heptane. It distilled at 165–166.5°C. at 751 mm.

$$D_4^{20} = 0.7354 \qquad\qquad N_D^{20} = 1.4126$$

0.1012 gm. substance: 0.3140 gm. CO_2 and 0.1420 gm. H_2O.

$C_{10}H_{22}$. Calculated. C 84.51, H 15.49, M_D^{20} 48.38.

Found.   " 84.60, " 15.70, " 48.12.

2-Butyl-Hexanol.—2-Butyl-hexanol was prepared exactly as described by Levene and Cretcher.[2] It boiled at 210–212°C. at 731 mm.

2-Butyl-Hexyl Iodide.—The alcohol was heated at 180°C. for 1 hour with iodine and red phosphorus, washed, dried, and distilled. The iodide boiled at 115–117°C. at 8 mm. Levene and Cretcher[2] found a boiling point of 124–125°C. at 13 mm.

0.2070 gm. substance: 0.1830 gm. AgI (Carius).
$C_{10}H_{21}I$. Calculated. I 47.39.
Found. " 47.76.

5-Methyl-Nonane.—2-Butyl-hexyl iodide was reduced with zinc dust in glacial acetic acid. The process has already been described. The hydrocarbon boiled at 164–166°C. at 755 mm. The boiling point found by Levene and Cretcher[2] was 165°C. The yield was 80 per cent of the theoretical.

$D_4^{20} = 0.7319$ $N_D^{20} = 1.4116$
0.1116 gm. substance: 0.3452 gm. CO_2 and 0.1574 gm. H_2O.
$C_{10}H_{22}$. Calculated. C 84.51, H 15.49, M_D^{20} 48.38.
Found. " 84.35, " 15.78, " 48.24.

4-Butyl-Octanol.—The procedure described by Levene and Cretcher[2] was followed for the conversion of 2-butyl-hexyl iodide into 4-butyl-octanol. The alcohol distilled completely at 127–130°C. at 8 mm.

4-Butyl-Octyl Iodide.—This iodide was prepared by heating the alcohol with iodine and red phosphorus at 180°C. It boiled at 145–146°C. at 10 mm. Levene and Cretcher[2] report 143°C. at 8 mm. The yield was 92 per cent of the theoretical.

0.2096 gm. substance: 0.1663 gm. AgI (Carius).
$C_{12}H_{25}I$. Calculated. I 42.91.
Found. " 42.87.

5-Propyl-Nonane.—4-Butyl-octyl iodide was reduced with zinc dust in glacial acetic acid. The method has already been described. The 5-propyl-nonane boiled at 204–205°C. at 763 mm. The yield was 75 per cent of the theoretical.

$D_4^{20} = 0.7559$ $N_D^{20} = 1.4228$
0.1004 gm. substance: 0.3112 gm. CO_2 and 0.1382 gm. H_2O.
$C_{12}H_{26}$. Calculated. C 84.71, H 15.29, M_D^{20} 57.62.
Found. " 84.52, " 15.40, " 57.25.

Diethyl Butyl-Malonate.—This ester was prepared by the condensation of malonic ester and butyl iodide in the usual manner. It boiled at 122°C. at 12 mm. Adams and Marvel[6] found a boiling point of 144–145°C. at 40 mm.   The yield was 75 per cent of the theoretical.

$$D_4^{20} = 0.9745 \qquad\qquad N_D^{20} = 1.4222$$

0.0988 gm. substance: 0.2209 gm. CO_2 and 0.0842 gm. H_2O.

$C_{11}H_{20}O_4$.   Calculated.   C 61.11, H 9.26, M_D^{20} 56.31.

Found.   " 60.97, " 9.53, "   56.35.

Diethyl Butyl-Heptyl-Malonate.—In the usual manner, diethyl butyl-malonate (1 equivalent) was condensed with heptyl iodide (1.1 equivalents).   The new ester boiled at 177–178°C. at 12 mm. The yield was 90 per cent of the theoretical.

$$D_4^{20} = 0.9318 \qquad\qquad N_D^{20} = 1.4366$$

0.1002 gm. substance: 0.2521 gm. CO_2 and 0.1014 gm. H_2O.

$C_{19}H_{34}O_4$.   Calculated.   C 68.79, H 10.83, M_D^{20} 88.63.

Found.   " 68.61, " 11.32, " · 88.22.

Butyl-Heptyl-Malonic Acid.—The ester was saponified by heating its alcoholic solution over night with a large excess of sodium hydroxide.   The acid crystallized from dilute acetone in fine needles which melted at 117°C.

0.1014 gm. substance: 0.2420 gm. CO_2 and 0.0924 gm. H_2O.

$C_{14}H_{26}O_4$.   Calculated.   C 65.12, H 10.08.

Found.   " 65.08, " 10.19.

2-Butyl-Nonylic Acid.—Butyl-heptyl-malonic acid was heated at 180°C. as long as carbon dioxide was evolved and the residue distilled.   2-butyl-nonylic acid boiled at 179°C. at 13 mm.

$$D_4^{20} = 0.8860 \qquad\qquad N_D^{20} = 1.4403$$

0.0993 gm. substance: 0.2661 gm. CO_2 and 0.1098 gm. H_2O.

$C_{13}H_{26}O_2$.   Calculated.   C 72.90, H 12.15, M_D^{20} 63.77.

Found.   " 73.08, " 12.37, "   63.69.

Ethyl 2-Butyl-Nonylate.—The acid was esterified by boiling its alcohol solution with sulfuric acid.   The ester distilled at 115°C. at 1 mm.

[6] Adams, R., and Marvel, C. S., *J. Am. Chem. Soc.*, 1920, xlii, 310.

$$D_4^{20} = 0.8560 \qquad\qquad N_D^{20} = 1.4290$$

0.0980 gm. substance: 0.2674 gm. CO_2 and 0.1088 gm. H_2O.

$C_{15}H_{30}O_2$.   Calculated.   C 74.38, H 12.38, M_D^{20} 73.42.

Found.   " 74.40, " 12.42, " 72.89.

2-Butyl-Nonanol.—The ester was reduced in the usual manner. The yield of alcohol was 72 per cent of the theoretical.   Practically all the remainder was recovered as 2-butyl-nonylic acid.   The alcohol boiled at 112–114°C. at 0.5 mm.

$$D_4^{20} = 0.8359 \qquad\qquad N_D^{20} = 1.4430$$

0.1013 gm. substance: 0.2899 gm. CO_2 and 0.1276 gm. H_2O.

$C_{13}H_{28}O$.   Calculated.   C 78.00, H 14.00, M_D^{20} 63.76.

Found.   " 78.06, " 14.09, "   63.43.

2-Butyl-Nonyl Iodide.—2-Butyl-nonanol was heated for 1 hour at 180°C. with iodine and red phosphorus.   The product was washed with sodium thiosulfate, dried, and distilled.   It boiled at 121–123°C. at 0.5 mm.

0.2116 gm. substance: 0.1602 gm. AgI (Carius).

$C_{13}H_{27}I$.   Calculated.   I 40.97.

Found.   " 40.90.

5-Methyl-Dodecane.—This hydrocarbon was prepared by the reduction of the 2-butyl-nonyl iodide with zinc dust in glacial acetic acid.   It boiled at 225.5–227°C. at 758 mm.   The yield was 91 per cent of the theoretical.

$$D_4^{20} = 0.7576 \qquad\qquad N_D^{20} = 1.4244$$

0.1084 gm. substance: 0.3354 gm. CO_2 and 0.1516 gm. H_2O.

$C_{13}H_{28}$.   Calculated.   C 84.78, H 15.22, M_D^{20} 62.23.

Found.   " 84.37, " 15.64, "   62.02.

Oxidation of 5-Methyl-Nonane.—The procedure for the oxidation of the hydrocarbon was developed from that described by Levene and Cretcher.[2]   The material (5 gm.) was shaken with 1,500 cc. of a solution containing 22.5 gm. each of potassium permanganate and potassium hydroxide at 37°C.   The shaking was continued for 16 hours.   Hydrogen peroxide was then added until the excess of permanganate was decomposed and the precipitated manganese dioxide filtered off.   The volatile fatty acids were separated from the filtrate by acidifying and distilling in a

current of steam as long as the distillate was acid to litmus.   The acids were neutralized with sodium hydroxide and concentrated under diminished pressure to a volume of about 15 cc.

Since a preliminary experiment showed that the mixture of acids obtained could not be easily separated by crystallization of the silver salts, the procedure recommended by Crowell[7] for the separation of butyric and acetic acids was tested in the following manner: 1 gm. of a mixture of 2 parts of butyric and 1 part of acetic acids was diluted to 15 cc., acidified with concentrated hydrochloric acid, and washed into a separatory funnel with two 25 cc. portions of saturated calcium chloride solution, containing 20 gm. of potassium chloride per liter, and two 25 cc. portions of kerosene.   After shaking, the layers were separated and the kerosene washed with 10 cc. of fresh calcium chloride solution.   The kerosene was then extracted with sodium hydroxide, the alkaline solution acidified and extracted with ether.   The ethereal solution was treated with a few drops of ammonium hydroxide and allowed to evaporate over night.   The residue should contain the ammonium salt of the butyric acid.

The calcium chloride solution was also extracted with ether and the ammonium salt prepared in the same manner.   The residue should contain ammonium acetate.

These ammonium salts were washed separately into flasks and treated with saturated silver nitrate solution.   The precipitates formed were crystallized from small amounts of water and analyzed.

Butyrate fraction:
  0.1422 gm. substance on ignition: 0.0790 gm. Ag.
                    $C_4H_7O_2Ag$.   Calculated.   Ag 55.4.
                                Found.   "   55.6.

Acetate fraction:
  0.2216 gm. substance on ignition: 0.1370 gm. Ag.
                    $C_2H_3O_2Ag$.   Calculated.   Ag 64.7.
                                Found.   "   64.8.

It is apparent that butyric acid can be obtained in a pure state from such a mixture but that acetic acid will be contaminated by a comparatively large proportion of the higher fatty acid.

The mixture of salts of the fatty acids obtained from the oxidation of 20 gm. of 5-methyl-nonane was therefore concentrated

[7] Crowell, R. D., *J. Am. Chem. Soc.*, 1918, xl, 453.

$C_2H_3O_2Ag.$ Calculated.   Ag 64.7.
$C_3H_5O_2Ag.$ "   " 59.7.
$C_4H_7O_2Ag.$ "   " 55.4.
              Found, Fraction   I.   " 61.3.
                   "   "   II. " 59.2.
                   "   "   III. " 58.2.
                   "   "   IV. " 60.5.
                   "   "   V.   " 62.9.

In order to determine the amount of destruction of the fatty acids by permanganate, both acetic and butyric acids were separately subjected to the conditions of the oxidation of the hydrocarbon. Acetic acid was not attacked, since there was no loss of permanganate in 24 hours. The butyric acid was slowly oxidized.

1 gm. of butyric acid was shaken with 1,500 cc. of alkaline permanganate for 16 hours at 37°C. and the fatty acids produced, isolated as before.

The butyrate fraction weighed 0.15 gm.

0.1004 gm. substance on ignition: 0.0553 gm. Ag.
              $C_4H_7O_2Ag.$ Calculated. Ag 55.4.
                         Found.   " 55.1.

From the acetate fraction 0.4 gm. were recovered: Fraction I, 0.3 gm.; and II, 0.1 gm.

Fraction I. 0.1004 gm. substance on ignition: 0.0621 gm. Ag.
   "   II. 0.1047 "   "   "   "   0.0605 "   "
              $C_2H_3O_2Ag.$ Calculated.   Ag 64.7.
                         Found, Fraction I.   " 61.9.
                              "   "   II. " 57.8.

STUDIES IN INORGANIC METABOLISM.

I. INTERRELATIONS BETWEEN CALCIUM AND MAGNESIUM METABOLISM.*

By L. JEAN BOGERT and ELIZABETH J. McKITTRICK.

(*From the Department of Food Economics and Nutrition, Kansas State Agricultural College, Manhattan.*)

Although considerable data have been gathered on the metabolism of the ash constituents, the literature concerning the interrelations between calcium and magnesium metabolism is very limited. This experiment was undertaken to ascertain whether an increased intake of calcium would affect the urinary and fecal excretion of magnesium and *vice versa*, thus showing interrelations between the metabolism of these two elements.

The injection of calcium salts, according to observations of Mendel and Benedict on dogs, cats, and rabbits (1), was followed by increased elimination of magnesium in the urine and similarly the injection of a magnesium salt increased the output of urinary calcium (2). Givens (3) found that the ingestion by human subjects of calcium in the form of raw milk, dried milk, or calcium lactate (about 1.5 to 5 gm.) caused an increased urinary calcium, while the ingestion of magnesium citrate (1 to 2.5 gm.) apparently had no effect on the urinary magnesium. Calcium lactate was less effective than milk in this respect. Underhill, Honeij, and Bogert (4) found that the ingestion of calcium lactate (2 gm.) increased the urinary calcium but not the urinary magnesium, while the same amount of magnesium citrate taken by mouth increased the urinary magnesium but not the urinary calcium.

Malcolm (5) has presented evidence to show that ingestion of soluble magnesium salts causes a loss of calcium in adult dogs and

363

hinders its deposition in young rats, while soluble calcium salts do not in the same way promote the excretion of magnesium. Underhill, Honeij, and Bogert (4) found evidence to show that, with normal human subjects in negative calcium balance, calcium lactate taken by mouth causes the balance to become positive. The negative balance was resumed upon the withdrawal of the added calcium. The increased calcium intake showed little or no influence upon the magnesium balance. Increasing the intake of magnesium changed a slightly negative magnesium balance to a strongly positive one without much influence upon the calcium balance.

Since conclusions as to interrelations between calcium and magnesium metabolism must be based upon experiments in which the intake has been carefully controlled and the urinary and fecal output of these two elements has been accurately determined, there seemed need of more extended metabolic studies in this field. These investigations were carried out on human subjects in the hope that the evidence thus obtained would be of greater usefulness and interest than if lower animals had been used as subjects.

EXPERIMENTAL.

Four healthy young women, two teachers, one student, and one secretary, lived for 16 days on an absolutely uniform diet. They were weighed at the beginning of the experiment and at the end of each 4 day period. A record was kept of any conditions, such as digestive disturbances or the menstrual period, which might affect the experiment. A mixed diet was selected which was expected to satisfy the requirements for energy, protein, and ash constituents.

Sherman[1] has pointed out from the results of nearly 100 experiments, that calcium equilibrium can be maintained on amounts ranging from 0.27 to 0.82 gm., with an average of 0.45 gm. of calcium per 70 kilos of body weight per day. While the magnesium requirement has never been definitely stated, it is usually expected that a diet furnishing sufficient calcium will probably furnish sufficient magnesium, since both elements are found in about the same proportion in many foods.

[1] Sherman, H. C., *J. Biol. Chem.*, 1920, xliv, 21.

The experimental diets furnished 0.266 gm. of calcium and 0.275 gm. of magnesium per day for Subjects A and B, and 0.309 gm. of calcium and 0.292 gm. of magnesium for Subjects C and D.   It is regrettable that the calcium intake fell slightly below the theoretical requirement.   On analysis of the foods used, it was found that several of these foods contained a smaller percentage of calcium than had been expected from the average composition of foods published by Sherman.   The energy and protein contents of the diet were ample [2] and the actual intake of calcium was about 90 per cent of the theoretical requirement.

The probability that the loss of alkali earths through the intestine may be increased by the presence of large quantities of fat, is brought out by Givens.[3]   For this reason, the fat in the diet was kept low and uniform in amount.   Because of the possibility of its affecting the calcium output, the amount of sodium chloride was also kept low and uniform.

The diet consisted of bread, butter, lean beef, potatoes, rice, peanut butter, apples, milk, and sugar.   All the foods used, with the exception of the bread and meat, were purchased at the beginning of the experiment, to insure as uniform composition as possible.   The bread was bought daily from the same bakery.   The meat was prepared at the beginning of and twice during the experiment, and a composite sample was analyzed.   Very lean beef, from which practically all the fat and connective tissue were dissected, was used.   After having been ground and well mixed, it was divided into 100 gm. portions, wrapped in paraffin paper and kept in cold storage.   The potatoes were weighed after being baked and peeled.   The apples were cored before weighing. Each person's daily supply of rice was weighed dry and cooked with the day's supply of milk in an individual container in a pressure cooker.   Dried milk from the Merrell-Soule Company was used, in order to secure milk of uniform composition.   The milk powder was accurately weighed out and made up to a definite dilution with distilled water.   The dilution was chosen to approximate the percentage composition of fresh milk.   The butter, bought in quantity, was melted by heating for a short time to 40–45° C. and

[2] Subject D weighed more than Subject C but her energy requirement was known not to exceed 2,200 calories.

[3] Givens, M. H., *J. Biol. Chem.*, 1917, xxxi, 441.

allowed to stand until all the water and solids had settled out.
The pure fat was then siphoned off and filtered. The foods were
accurately weighed and the whole allowance was consumed.   As
far as possible the foods were eaten from the dishes in which they
were cooked to avoid loss in transfer.   Only distilled water was
used for drinking and for cooking.   Tea and coffee were used in
moderation, the calcium contained being considered negligible.
An effort was made to secure a fairly uniform intake of liquid
throughout the experiment.   Each subject consumed 4 gm. of
salt per day.

A calcium-low diet was consumed for 4 days prior to the begin-
ning of the experiment proper, and also at the close of the experi-

TABLE I.

Calcium and Magnesium Content of Foods Used in Diets.

Food.	Calcium.	Magnesium.
	per cent	per cent
Bread..	0.041	0.029
Beef...	0.008	0.028
Apples...	0.004	0.009
Potatoes...	0.004	0.035
Rice...	0.009	0.035
Peanut butter....................................	0.033	0.162
Milk...	0.096	0.016
Calcium lactate..................................	12.800	
Magnesium citrate................................		12.850

ment until the last collection of the feces was made.   The
experimental period proper was divided into four 4 day periods.
Period I represented normal calcium and magnesium intake.
Period II represented magnesium-high and calcium-normal intake,
the magnesium being raised by adding to the regular diet 6 gm. of
magnesium citrate in three equal doses daily. Period III was
exactly like Period I.   Period IV represented calcium-high and
magnesium-normal intake, the calcium being raised by adding to
the diet 6 gm. of calcium lactate in three equal doses daily.

The experimental periods were marked off in the feces by the
taking of carmine just before the first meal of each period.   Col-
lections were made in weighed pans, in which they were later dried
to constant weight.   The dried feces were combined in periods,

ground, and thoroughly mixed. The daily collections of urine were measured, made acid, filtered, and combined into periods. Calcium in the urine and feces was determined by the McCrudden method.[4] The magnesium determinations and the food analyses were done according to the Official Methods of Analysis of the Association of Official Agricultural Chemists.[5] The calcium and magnesium content of foods used in the daily diet is given in Table I. A summary of the daily food intake of the various subjects, together with the amounts of energy, protein, fat, calcium, and magnesium furnished by the diets, is contained in Table II.

TABLE II.

Daily Food Intake.

Food.	Subjects A and B.	Subjects C and D.
	gm.	*gm.*
Beef..	100.0	100.0
Bread.......................................	195.0	208.0
Apples......................................	477.0	477.0
Rice..	54.2	75.0
Potatoes....................................	149.5	149.5
Butter fat.................................	20.0	30.0
Peanut butter..............................	33.0	33.0
Milk.......................................	144.5	180.6
Sugar......................................	75.0	75.0

The diet for Subjects A and B furnished 2,000 calories, 62 gm. of protein, 48 gm. of fat, 0.266 gm. of calcium, and 0.275 gm. of magnesium.

The diet for Subjects C and D furnished 2,208 calories, 66 gm. of protein, 57 gm. of fat, 0.309 gm. of calcium, and 0.292 gm. of magnesium.

DISCUSSION.

All the subjects remained in good health and maintained their weight throughout the experiment. Subjects A and C had some trouble with constipation. The menstrual period was included for all subjects but apparently had no influence on the excretion of calcium or magnesium, which is in accordance with the more extended work of Sherman, Gillett, and Pope[6] on the monthly

[4] McCrudden, F. H., *J. Biol. Chem.*, 1909–10, vii, 83; 1911–12, x, 187.

[5] These analyses were done at the Agricultural Experiment Station of the Kansas State Agricultural College.

[6] Sherman, H. C., Gillett, L. H., and Pope, H. M., *J. Biol. Chem.*, 1918, xxxiv, 373.

metabolism of nitrogen, phosphorus, and calcium in healthy women.

In only one instance was any subject in positive calcium balance in either the first or third periods, but the negative balances were very small, indicating that the calcium requirement had been almost met. Judging from the total excretion and the amount of negative balance, the calcium requirements of the four subjects would seem to range from about 0.27 to 0.42 gm. per day. This corresponds closely to the theoretical requirements, based on Sherman's estimate of the average calcium requirement,[1] which ranged from 0.30 to 0.36 gm. per day.

On examination of the magnesium balances it is apparent that the magnesium requirement was met in every instance during the first or normal period. Based on total excretion and positive balances of the first period, the magnesium requirement of the four subjects would range from 0.10 to 0.20 gm. per day. This would seem to indicate that the magnesium requirement of the body may be somewhat lower than the calcium requirement.

Conclusions concerning normal magnesium metabolism can be based only on the first period, since the greatly increased intake of magnesium during the second period has very evidently caused an abnormally high magnesium excretion in Period III. On account of the possible interrelations between calcium and magnesium metabolism, it is impossible to consider the excretion of calcium in Period II or that of magnesium in Period IV as normal. Because of these facts, Period I has been taken as the normal period for comparison in drawing conclusions with regard to the experimental periods.

In considering the first period alone (see Tables III and IV), two of the four subjects show urinary magnesium to be less than urinary calcium, while the other two show urinary calcium to be less than urinary magnesium. Subjects A and B were consistent in the magnesium greater than calcium ratio throughout the experiment, with the exception of Subject B in the last period. This was the high calcium period and the ingestion of large doses of calcium probably was responsible for the disturbance of the ratio. Subject D shows urinary calcium higher than urinary magnesium in all four periods, while Subject C shows the same relation in all except the second and third periods, where the

urinary magnesium has been greatly increased by the ingestion of the large doses of magnesium in Period II. These results do not seem to be in agreement with the statement of Givens (3) that urinary calcium is greater than urinary magnesium normally in most cases, nor do they bear out the 1:1 ratio suggested by several writers. They tend to confirm the observations of Nelson and Burns (6), who conclude that either calcium or magnesium may be

TABLE III.

Average Daily Intake and Output of Calcium.

Subject.	Body weight.	Period.	Output.			Intake.	Balance.
			Urine.	Feces.	Total.		
	kj.		*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
A	47.3	Normal	0.064	0.177	0.241	0.266	+0.025
		Mg-high.	0.065	0.185	0.250	0.266	+0.016
		Normal.	0.072	0.229	0.301	0.266−	−0.035
		Ca-high.	0.105	0.926	1.031	1.035	+0.004
B	51.4	Normal.	0.071	0.245	0.316	0.266	−0.050
		Mg-high.	0.112	0.215	0.327	0.266	−0.061
		Normal.	0.077	0.215	0.292	0.266	−0.026
		Ca-high.	0.168	0.882	1.050	1.035	−0.015
C	56.1	Normal.	0.112	0.244	0.356	0.309	−0.047
		Mg-high.	0.169	0.269	0.438	0.309	−0.129
		Normal.	0.127	0.313	0.440	0.309	−0.131
		Ca-high.	0.167	0.599	0.766	1.078	+0.312
D	86.8	Normal.	0.167	0.205	0.372	0.309	−0.063
		Mg-high.	0.182	0.223	0.405	0.309	−0.096
		Normal.	0.183	0.183	0.366	0.309	−0.057
		Ca-high.	0.246	0.786	1.032	1.078	+0.046

in excess in the urine, and whichever predominates does so consistently in each individual.

The fecal magnesium is less than the fecal calcium in all four cases during the first period, the ratio varying from 1:1.2 to 1:2.2. The total magnesium excretion is less than the total calcium excretion in all four cases, the ratio varying from 1:1.2 to 1:1.8. This supports the statement of Givens and Mendel (7) that calcium in the feces is always in excess of magnesium.

The ratio of the magnesium found in the urine to that found in the feces varies from 1:1.1 to 1:2.5, while the ratio of urinary calcium to fecal calcium varies from 1:1.2 to 1:3.4.   The magnesium shows quite a varied distribution but corresponds rather closely to the ratio of 1:2 as given by Underhill, Honeij, and Bogert (4), while the calcium distribution confirms their statement concerning a wide variation in the relation of calcium in the urine and feces of normal individuals.

TABLE IV.

Daily Intake and Output of Magnesium.

Subject.	Body weight.	Period.	Output.			Intake.	Balance.
			Urine.	Feces.	Total.		
	kg.		*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
A	47.3	Normal.	0.072	0.132	0.204	0.275	+0.071
		Mg-high.	0.217	0.385	0.602	1.046	+0.444
		Normal.	0.225	0.376	0.601	0.275	−0.326
		Ca-high.	0.123	0.195	0.318	0.275	−0.043
B	51.4	Normal.	0.100	0.112	0.212	0.275	+0.063
		Mg-high.	0.184	0.617	0.801	1.046	+0.245
		Normal.	0.126	0.187	0.313	0.275	−0.038
		Ca-high.	0.076	0.164	0.240	0.275	+0.035
C	56.1	Normal.	0.080	0.113	0.193	0.292	+0.099
		Mg-high.	0.188	0.633	0.821	1.063	+0.242
		Normal.	0.182	0.271	0.453	0.292	−0.161
		Ca-high.	0.118	0.129	0.247	0.292	+0.045
D	86.8	Normal.	0.070	0.176	0.246	0.292	+0.046
		Mg-high.	0.153	0.753	0.906	1.063	+0.157
		Normal.	0.090	0.175	0.265	0.292	+0.027
		Ca-high.	0.067	0.190	0.257	0.292	+0.035

The percentage of the calcium intake excreted through the feces in the first three periods ranged from 59 to 101 with an average of 78 per cent.   The percentage of calcium intake excreted through the urine in the first three periods ranged from 24 to 59, with an average of 40 per cent (Table V).   The large percentage of calcium excreted by way of the urine is noteworthy, especially in Subjects C and D.   The percentages of magnesium intake

excreted by way of the urine and feces in the different periods are given in Table VI.   Due to the fact that the magnesium excretion was disturbed in a number of cases for several periods by the large doses of magnesium citrate ingested in the second period, it seems unwise to draw conclusions as to the normal distribution of magnesium between urine and feces from these figures.

The addition of 6 gm. of magnesium citrate to the regular diet during the second period increased the urinary and fecal magne-

TABLE V.

Distribution of Calcium in Urine and Feces.

Subject.		Period I		Period II.		Period III.		Period IV.	
		gm.	per cent	gm.	per cent	gm.	per cent	gm.	per cent
A	Ca intake.	0.266		0.266		0.266		1.035	
	Urine.	0.064	24.1	0.065	24.4	0.072	27.0	0.105	10.1
	Feces.	0.177	66.5	0.185	69.6	0.229	86.1	0.926	89.5
	Total.	0.241	90.6	0.250	94.0	0.301	113.1	1.031	99.6
B	Ca intake.	0.266		0.266		0.266		1.035	
	Urine.	0.071	26.7	0.112	42.1	0.077	28.9	0.168	16.2
	Feces.	0.245	92.1	0.215	80.8	0.215	80.8	0.882	85.2
	Total.	0.316	118.8	0.327	122.9	0.292	109.7	1.050	101.4
C	Ca intake.	0.309		0.309		0.309		1.078	
	Urine.	0.112	36.2	0.169	54.7	0.127	41.1	0.167	15.5
	Feces.	0.244	78.9	0.269	87.0	0.313	101.3	0.599	55.5
	Total.	0.356	115.1	0.438	141.7	0.440	142.4	0.766	71.0
D	Ca intake.	0.309		0.309		0.309		1.078	
	Urine.	0.167	54.0	0.182	58.9	0.183	59.2	0.246	22.8
	Feces.	0.205	66.3	0.223	72.2	0.183	59.2	0.786	72.9
	Total.	0.372	120.3	0.405	131.1	0.366	118.4	1.032	95.7

sium in all four cases to quite an appreciable degree.   The percentage of increase, based on the first or normal period, varied from 80 to 200 in the urine and from 190 to 460 in the feces.   This same period of high magnesium intake showed an increase in the urinary calcium in three out of four cases, the increase ranging from 9 to 57 per cent.   The fecal calcium was also increased by the added magnesium in three out of four cases, but to a lesser degree, the percentage increase ranging from 4.5 to 10.   The total calcium excretion of this period, however, was increased in all four cases,

the percentage of increase varying from 3.3 to 23 per cent.   It is interesting to note that Subject B, who showed a slight loss in fecal calcium during this period of high magnesium intake, showed the largest percentage gain in urinary calcium during this same period, indicating that, in this subject, the increased calcium output was excreted chiefly through the urine. Contrary to the results of Givens (3), who was unable to get an increased urinary output of magnesium by the ingestion of magnesium citrate, the results of

TABLE VI.

Distribution of Magnesium in Urine and Feces.

Subject.		Period I.		Period II.		Period III.		Period IV.	
		gm.	per cent	gm.	per cent	gm.	per cent	gm.	per cent
A	Mg intake.	0.275		1.046		0.275	·	0.275	
	Urine.	0.072	26.2	0.217	20.7	0.225	81.8	0.123	44.7
	Feces.	0.132	48.0	0.385	36.8	0.376	136.7	0.195	70.9
	Total.	0.204	74.2	0.602	57.5	0.601	218.5	0.318	115.6
B	Mg intake.	0.275		1.046		0.275		0.275	
	Urine.	0.100	36.3	0.184	17.6	0.126	45.8	0.076	27.6
	Feces.	0.112	40.7	0.617	58.9	0.187	68.0	0.164	59.6
	Total.	0.212	77.0	0.801	76.5	0.313	113.8	0.240	87.2
C	Mg intake.	0.292		1.063		0.292		0.292	
	Urine.	0.080	27.4	0.188	17.7	0.182	62.3	0.118	42.9
	Feces.	0.113	38.6	0.633	59.5	0.271	92.7	0.129	46.9
	Total.	0.193	66.0	0.821	77.2	0.453	155.0	0.247	89.8
D	Mg intake.	0.292		1.063		0.292		0.292	
	Urine.	0.070	23.9	0.153	14.4	0.090	30.7	0.067	24.3
	Feces.	0.176	60.2	0.753	70.8	0.175	59.9	0.190	69.1
	Total.	0.246	84.1	0.906	85.2	0.265	90.6	0.257	93.4

this experiment show an increase in every case (Table IV).   This difference can perhaps be accounted for by the fact that the doses of magnesium which Givens gave were comparatively small (1 to 2.5 gm.).   However, Underhill, Honeij, and Bogert (4) found an increased urinary magnesium after the ingestion of only 2 gm. of magnesium citrate.

With regard to the effects of the addition of calcium lactate to the experimental diet, in doses of 2 gm. each three times a day during the last period, it can be seen by consulting Table III, that

the urinary and fecal calciums were raised very noticeably in every case. The urinary increase ranged from 31 to 128 per cent above that of the first or normal period, and the fecal increase from 145 to 432 per cent. It is a little difficult to interpret the effects of the added calcium intake of Period IV on the magnesium output of this period, because the high magnesium intake of Period II seems to have kept both the urinary and fecal magnesium output abnormally high in Period III as well as in Period II, especially in Subjects A and C. However, it is evident that the urinary magnesium in Period IV was decidedly increased above that of Period I in Subjects A and C, remained practically constant for Subject D, and was somewhat lowered for Subject B. The probable interrelation between calcium intake and magnesium output is seen more clearly in the fecal magnesium which showed an increase over that of Period I in every case, while the total magnesium output also showed an increase in every case, ranging from 4.5 to 56 per cent. These results confirm the work of Mendel and Benedict (2), who found the injection of magnesium salts to cause increased urinary calcium, and agree in part with their results that the injection of calcium salts causes increased urinary magnesium (1). Neither Givens (3) nor Underhill, Honeij, and Bogert (4) were able to produce increased urinary magnesium by the addition of calcium lactate to the diet.

CONCLUSIONS.

1. *The addition of 6 gm. of magnesium citrate per day to the experimental diet increased the urinary and fecal magnesium* in all four cases. *The added magnesium also increased the urinary and fecal calcium* in three out of four cases, *and the total calcium excretion* in all four cases.

2. *The addition of 6 gm. of calcium lactate per day to the experimental diet lead to decided increases in both urinary and fecal calcium in all four cases.* It seemed to result in increased urinary magnesium in two instances and in a slightly increased fecal and total magnesium output in all four subjects, although possible after effects of the magnesium citrate taken in Period II make it impossible to draw definite conclusions as to the influence of the calcium lactate upon magnesium metabolism.

BIBLIOGRAPHY.

1. Mendel, L. B., and Benedict, S. R., *Am. J. Physiol.*, 1909–10, xxv, 23.
2. Mendel, L. B., and Benedict, S. R., *Am. J. Physiol.*, 1909–10, xxv, 1.
3. Givens, M. H., *J. Biol. Chem.*, 1918, xxxiv, 119.
4. Underhill, F. P., Honeij, J. A., and Bogert, L. J., *J. Exp. Med.*, 1920, xxxii, 65.
5. Malcolm, J., *J. Physiol.*, 1904–05, xxxii, 183.
6. Nelson, C. F., and Burns, W. E., *J. Biol. Chem.*, 1916–17, xxviii, 237.
7. Givens, M. H., and Mendel, L. B., *J. Biol. Chem.*, 1917, xxxi, 435.

STUDIES IN INORGANIC METABOLISM.

II. THE EFFECTS OF ACID-FORMING AND BASE-FORMING DIETS UPON CALCIUM METABOLISM.

By L. JEAN BOGERT and ELIZABETH E. KIRKPATRICK.

(From the Department of Food Economics and Nutrition, Kansas State Agricultural College, Manhattan.)

Although the importance of calcium in nutrition and the common nutritional disturbances due to calcium deficiency are well established facts, the conditions which affect the retention of calcium in the body are imperfectly understood. One phase of this subject, which is still in the experimental stage, is the effect which acid and base have upon calcium retention. Several experiments have been performed adding acid or base to the diet, others in which acid-forming or base-forming rations were fed, but of the latter only a few were done quantitatively or with human subjects. Futhermore, the results obtained have often been of a conflicting or doubtful nature.

Steenbock, Nelson, and Hart (1) found that high ingestion of acid-forming foods appeared to cause decalcification of the bones in calves and swine, although such rations produced no serious effects upon growth and reproduction. Another experiment, showing the effect of an acid-forming diet, was carried on by Chalmers Watson,[1] who found that animals, which were fed on an exclusive meat diet, developed an appearance of delayed and imperfect ossification with increased vascularity in the bones. D. Forsyth[1] found that domestic fowls remained healthy after being fed from 11 months to 2 years upon meat, supplemented by lime; and rats fed solely on meat were reported by E. J. Spriggs[1] to have rough and abnormal coats, which improved almost to normal when lime was added to the diet. Rations, which did not provide

---

[1] Reviewed by Forbes (2).

enough mineral bases to neutralize the acids, caused malnutrition of the bones of swine in experiments by Forbes (2), while the addition of glycerophosphates of the same mineral bases overcame the the bad effect by neutralizing the excess acid. He attributed to the same cause the pathological conditions and decrease in growth of bone and muscle, which resulted when, in earlier experiments, he fed large amounts of water extract of wheat, which contained an excess of acid-forming mineral elements. He also called attention (2) to the experiments of Weiske in which prolonged feeding of cereals reduced the amount of calcium in the bones of herbivora.

Sherman (3) found that, when lean beef was added to a diet on which calcium equilibrium had been established, a negative calcium balance resulted. However, he did not attribute the loss of calcium to the preponderance of acid-forming food in the diet because of a previous experiment,[2] which did not indicate any definite relation between an excess of acid- or base-forming foods and the calcium needed for maintenance. In this experiment, one subject showed an average excretion of 0.08 gm. of calcium per day on a diet which yielded a strong preponderance of acid-forming elements, while on a "balanced" diet the same subject excreted 0.10 gm. of calcium per day.

Dubois and Stolte[3] were the first to study the effect of alkali on the retention of calcium in infancy. They believed that the favorable affect of Keller's malt soup upon calcium retention was due to its alkali content. The addition of alkali to milk was found by Sato (4), however, to produce an unfavorable effect upon infants. He gave malt extract alone with a beneficial effect upon calcium retention but, when he added a considerable amount of alkali to the malt extract, it seemed to have an unfavorable influence upon the calcium balance. He stated that probably ammonia is much more readily available than calcium for the neutralization of acids in the body.

EXPERIMENTAL.

The metabolism experiments described in this paper were carried out to investigate the effects of acid-forming and base-forming

[2] Sherman, H. C., Gillett, L. H., and Pope, H. M., *J. Biol. Chem.*, 1918, xxxiv, 373.

[3] Dubois, M., and Stolte, K., *Jahrb. Kinderh.*, 1913, lxxvii, 21.

diets upon calcium excretion in human subjects. The subjects were four normal women, three college students and one librarian. All of the food eaten was carefully weighed and its calcium content determined, while quantitative determinations were made of the amounts of calcium in the feces and urine. The technique of collection of excreta and of methods of analysis used will be found in the preceding paper,[4] which also contains descriptions of the foods used, their preparation, and weighing. The same foods constituted the diet of the subjects of this experiment, with the addition of peas and eggs. The canned peas were thoroughly drained before weighing. The eggs were boiled hard in the shell and the whites and yolks weighed separately.

The experimental period of 16 days was divided into four periods of 4 days each. It was preceded by a 4 day period of calcium-low diet; the same calcium-low diet was taken in the after period until the last marker appeared in the feces. In the first and third periods, the diets were selected so that the excess of base furnished by certain foods was practically balanced by the excess of acid supplied by the other foods. The diet of the second period consisted almost entirely of base-forming foods and that of the fourth, of acid-forming foods. The data for the acidity or alkalinity of the foods were taken from Sherman's tables,[5] which express the excess of acid or base in cubic centimeters of normal solution. The balanced diet consisted of beef, peanut butter, bread, rice, potatoes, milk, apples, butter fat, and sugar; the base diet, of apples, milk, peas, potatoes, peanut butter, and sugar; and the acid diet of bread, rice, eggs, milk, butter fat, and sugar.

The diets were planned to meet the requirements for energy, protein, and calcium and to yield uniform amounts of energy, protein, calcium, and fat in all four periods. The calcium requirements of the subjects, estimated from Sherman's figure (3) of 0.45 gm. per day per 70 kilos of body weight, ranged from 0.31 to 0.37 gm. Unfortunately, the amount of calcium in some of the foods (see Table I) was found on analysis to vary from the average figures given by Sherman,[6] so that the intake of calcium not only

[4] Bogert, L. J., and McKittrick, E. J., *J. Biol. Chem.*, 1922, liv, 363.

[5] Sherman, H. C., and Gettler, A. O., *J. Biol. Chem.*, 1912, xi, 329.

[6] Sherman, H. C., Chemistry of food and nutrition, New York, 2nd edition, 1919, 421.

was somewhat below the theoretical requirement, but was not as nearly constant as had been expected. The average percentages of the theoretical calcium requirement furnished by the diets were as follows:

per cent

Balanced diet, Periods I and III............................... 80.6
Basic diet, Period II.. 70.4
Acid diet, Period IV.. 91.6

TABLE I.

Calcium Content of Foods Used in Diets.

Food.	Calcium.
	per cent
Bread..	0.0410
Beef..	0.0078
Apples..	0.004
Potatoes, baked..	0.004
Rice, uncooked..	0.009
Peanut butter..	0.033
Peas..	0.032
Egg yolk..	0.1029
Egg white..	0.0092
Milk, Klim (whole milk)................................	0.0958

TABLE II.

Daily Food Intake.

Food.	Subjects E and F.			Subjects G and H.		
	Periods I and III.	Period II.	Period IV.	Periods I and III.	Period II.	Period IV.
	gm.	gm.	gm.	gm.	gm.	gm.
Beef................	100.0			100.0		
Egg white...........			130.0			130.0
Egg yolk............			120.0			97.0
Peanut butter........	48.0	112.0		45 0	105.0	
Butter fat..........	25.8		13 0	16.0		10.0
Bread...............	175.5		160.0	135.0		160.0
Rice................	71.0		150 0	86.0		150.0
Potato..............	82.0	350 0		135.0	290.0	
Sugar...............	80.0	116.0	135.0	72.0	101.0	107.0
Apples..............	477.0	510.0		432.0	422.0	
Peas................		307.6			307.6	
Milk................	180.0	95 0	120.0	140.0	64.5	100.0

TABLE III.

Composition of Diets.

Period.	Calories.	Protein.	Fat.	Calcium.	Excess* of	
					Base.	Acid.
Subjects E and F.						
		gm.	*gm.*	*gm.*	*cc.*	*cc.*
I and III	2,221.8	67.6	61.2	0.297	29.46	31.61
II	2,182.5	65.1	59.4	0.260	59.90	4.73
IV	2,185.9	65.9	60.7	0.319	2.27	62.14
Subjects G and H.						
I and III	1,968.0	63.5	47.8	0.242	28.27	30.09
II	1,946.3	59.8	54.5	0.223	49.99	4.43
IV	1,944.8	61.4	48.9	0.287	1.79	56.31

* Potential acidity or alkalinity is expressed in cubic centimeters of normal solution.

TABLE IV.

Average Daily Intake and Output of Calcium.

Subject.	Body weight.	Period.	Output.			Intake.	Balance.
			Urine.	Feces.	Total.		
	kg.		*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
E	55.1	Balanced.	0.086	0.303	0.389	0.297	−0.092
		Basic.	0.046	0.344	0.390	0.260	−0.130
		Balanced.	0.075	0.223	0.298	0.297	−0.001
		Acid.	0.137	0.338	0.475	0.319	−0.156
F	57.4	Balanced.	0.137	0.236	0.373	0.297	−0.076
		Basic.	0.116	0.241	0.357	0.260	−0.097
		Balanced.	0.183	0.157	0.340	0.297	−0.043
		Acid.	0.231	0.197	0.428	0.319	−0.109
G	48.8	Balanced.	0.074	0.276	0.350	0.242	−0.108
		Basic.	0.039	0.275	0.314	0.223	−0.091
		Balanced.	0.123	0.303	0.426	0.242	−0.184
		Acid.	0.086	0.240	0.326	0.287	−0.039
H	48.3	Balanced.	0.101	0.300	0.401	0.242	−0.159
		Basic.	0.065	0.273	0.338	0.223	−0.115
		Balanced.	0.097	0.200	0.297	0.242	−0.055
		Acid.	0.138	0.207	0.345	0.287	−0.058

The detailed composition of the diets used is shown in Tables II and III.   The data obtained from the analysis of the excreta are presented in Tables IV, V, and VI.

DISCUSSION.

All the subjects were in good health and continued so throughout the experiment with the exception of Subject G, who experienced some digestive difficulty as a result of the diet in Period II.   Their

TABLE V.

Relative Amounts of Calcium Excreted in Urine and Feces.

Subject.	Period.	Urine.	Feces.
		per cent	per cent
E	I	22.3	77.7
	II	11.9	88.1
	III	25.1	74.9
	IV	28.8	71.2
F	I	36.6	63.4
	II	32.5	67.5
	III	53.8	46.2
	IV	53.9	46.1
G	I	21.0	79.0
	II	12.3	87.7
	III	28.7	71.3
	IV	26.4	73.6
II	I	25.1	74.9
	II	19.3	80.7
	III	32.5	67.5
	IV	40.7	59.3

weights remained almost constant.   The menstrual period for all four subjects was included during the experiment, but did not seem to affect the calcium output.

Owing to differences between the actual percentages of calcium in the foods used and the average percentages upon which the dietaries were planned, the intake was lower than the theoretical requirement and all the subjects showed negative calcium balance throughout the experiment.   The most unfortunate effect of the

variations in the calcium composition of the foods used was the fact that the total intake of calcium varied instead of being practically constant, as planned. The calcium intake was highest during the acid-forming diets, while the base-forming diets furnished considerably less calcium than the other diets. As has been previously noted, the acid-forming diets furnished about 90 per cent, the balanced diets about 80 per cent, and the

TABLE VI.

Comparison of Calcium Deficiency of the Diet With the Calcium Balance.

Subject.	Period.	Require-ment.	Intake.	Output.	Balance.	Deficiency in diet.	Balance minus deficiency of diet.
		gm.	*gm.*	*gm.*	*gm.*	*gm*	*gm.*
E	I	0 354	0.297	0.389	− 0 092	0 057	− 0 035
	II		0.260	0 390	− 0.130	0 694	− 0.036
	III		0.297	0 298	− 0 001	0 057	+ 0 056
	IV		0.319	0.475	− 0 156	0 035	− 0.121
F	I	0 369	0 297	0.373	− 0 076	0 072	− 0 004
	II		0.260	0 357	− 0 097	0 109	+ 0.012
	III		0.297	0.340	− 0.043	0 072	+ 0.029
	IV		0 319	0 428	− 0 109	0 050	− 0 059
G	I	0 311	0 242	0.350	− 0 108	0 069	− 0.039
	II		0.223	0.314	− 0 091	0.088	− 0 003
	III		0 242	0 426	− 0.184	0.069	− 0 115
	IV		0.287	0.326	− 0 039	0 024	− 0 015
H	I	0.310	0.242	0.401	− 0.159	0.068	− 0 094
	II		0.223	0.338	− 0.115	0 087	− 0 028
	III		0.242	0.297	− 0 055	0 068	+ 0 013
	IV		0.287	0.345	− 0 058	0 023	− 0 035

base-forming diets about 70 per cent of the theoretical calcium requirement.

In Table VI is shown the result of subtracting the calcium deficiency of the diet from the calcium balance. Some of the discussion of data is based upon the theory that this difference between the dietary calcium deficiency and the calcium balance represents what the balance might have been if the theoretical calcium requirement had been met. There is also the possibility that the calcium furnished by the different diets may not have

been equally well assimilated. This might be expected especially in the base-forming diets of Period II, since peas formed so large a part of these diets and the nitrogen of this food has been shown to be poorly utilized. Although the number of stools was increased during this period and the feces were somewhat more loose, no actual diarrhea was produced (with the possible exception of Subject G), and the calcium balances do not warrant the conclusion that the calcium of the food was more poorly utilized on this diet.

It might have been expected, since the diets for Periods I and III were the same, that the calcium balances would have been approximately the same in these two periods, but reference to Table IV will show that three subjects had appreciably smaller negative calcium balances in the third than in the first period. The reason for this difference may be that during the first period, in spite of the low calcium fore period of 4 days, the body was not yet adjusted to the small calcium intake, while by the third period calcium metabolism had more nearly reached a minimum amount. On the other hand, the lower output of calcium on the balanced diet of Period III might be attributed to after effects of the base-forming diet of Period II, when the calcium intake was lower. Because of the impossibility of determining which of the two periods on a balanced diet represents a true normal for purposes of comparison, it was thought wiser in interpreting the data to compare the calcium excretion and balances in the period on the base-forming diet (Period II) and the period on the acid-forming diet (Period IV) with those of the balanced period immediately preceding each, rather than with an average of Periods I and III.

The urinary calcium was considerably lower, in every case, in the period on the base-forming diet than in the preceding period on a balanced diet (Table IV). Moreover, the amount of calcium excreted in the urine during the final period, when an acid-forming diet was consumed, was decidedly higher than that excreted in the preceding period, except in the case of Subject G. Since this subject had difficulty in recovering from the digestive disturbance caused by the diet of the second period, the high calcium excretion which she showed in Period III may be attributable to after effects of the previous diet. The large amount of the urinary calcium of Subject G in Period III may be partly accounted for by a greatly increased volume of the urine in this period. In any

event, the abnormal condition of this subject during the latter half of the experimental period seemed to offer probable explanation of the exception which she showed to the usual effects of an acid-forming diet, as shown in the other three subjects.

The effects of the acid-forming and base-forming diets upon fecal calcium were not so apparent as their influence upon the urinary calcium. In Subjects E and F, the calcium output in the feces was higher in both the basic and acid periods than it was in the periods on the balanced diet which preceded each. Subject G had almost the same fecal calcium in the basic period as in the balanced, but the calcium in the feces was decreased during the acid period. Subject H was like Subjects E and F in that the output of calcium rose in the acid period, but differed from them in showing a decrease instead of an increase in fecal calcium in the period on the base-forming diet.

Although the actual amount of calcium excreted in the feces was not so uniformly affected by the acid- and base-forming diets, it is clear that the base-forming diet tended to divert calcium from the urine to the feces, while the acid-forming diet had the opposite effect of increasing the percentage of the calcium output excreted in the urine and decreasing it in the feces. Table V shows the percentage distribution of calcium output between the urine and feces. It will be seen that, in every case, the per cent of calcium excreted in the urine was decreased and the per cent excreted by the feces increased in the second period. The effect of the acid-forming diet in increasing the per cent of calcium excreted by way of the urine and decreasing fecal calcium is clear in two instances. In the other two subjects, this effect is only to be noted in case the figures for Period IV are compared with those of Periods I or II. These facts, and the additional observation, that the base-forming diet had a greater effect in changing the route of calcium excretion than the acid-forming diet, are apparent from the following figures compiled from Table V:

Calcium.	Balanced diet.		Base-forming diet.		Acid-forming diet.	
	Range.	Average.	Range.	Average.	Range.	Average.
	per cent	per cent	per cent	per cent	per cent	per cent
Urinary.........	21–54	30	12–32	19	26–54	37
Fecal...........	46–79	70	67–88	81	46–74	63

It is interesting to note that the amount of calcium excreted by way of the urine may be even greater than the amount in the faces. The average distribution between urine and feces on a balanced diet corresponds very closely to that often given (3 : 7).

The effects of the acid-forming and base-forming diets upon the total calcium output were similar to their effects upon urinary calcium, since the basic period showed a decrease and the acid period an increase in the total calcium excretion in three out of four cases.   In Subject E, the output in the basic period was the same as in the preceding balanced period, and the output in Subject G, who was known to be in an abnormal condition, was lowered in the acid period.

Since the intake of calcium was appreciably lower in the basic period and somewhat higher in the acid period, the calcium balance must be given more weight than the amount excreted.   The negative calcium balances were increased in both the acid and basic periods in two subjects, while the other two cases showed diminished negative calcium balances in the basic and acid periods, with the exception of Period IV, Subject H, where the balance remained the same as in the preceding period.   However, the variation in the calcium deficiency of the diet must be taken into account in interpreting the calcium balances.   These figures are tabulated in Table VI and show that, not only can the increase in the negative calcium balance in two cases in Period II probably be accounted for entirely by the increased deficiency of the diet, but in the other two cases the diminution in calcium output was more than sufficient to compensate for the diminished intake, so that the negative calcium balance was actually decreased on the basic diet which afforded less calcium.   On the other hand, the considerably increased negative calcium balance shown by Subjects E and F during the fourth period cannot be accounted for at all by a corresponding increase in the deficiency of the diet, since the diet was richer in calcium in this period than in any other. Subject H showed an increased total excretion of calcium in Period IV, part of which might be accounted for by increased calcium intake.   Subject G offered an exception in that she had a decreased negative calcium balance in Period IV.   While not conclusive, the above noted facts seem to show a possible tendency toward diminished calcium excretion on the base-forming diet and a rather well

marked tendency toward increased excretion of calcium on the acid-forming diet.

The effect of an acid-forming diet in increasing urinary calcium, which is shown in these experiments, is in accord with the results of earlier experiments where acid was given as such. A number of investigators, whose work has been reviewed by Forbes (2) have demonstrated increased urinary calcium after giving hydrochloric and other acids. However, Stehle and McCarty (5) stated that ingestion of hydrochloric acid produced no appreciable effect upon urinary calcium in man. Stehle (6) found an increased excretion of calcium in dogs after the ingestion of hydrochloric acid, but Givens and Mendel (7) noted no increase of total calcium excretion under similar experimental conditions. The decrease of urinary calcium as a result of the base-forming diet is in contrast to the observation by Givens and Mendel (7), that 40 gm. of sodium bicarbonate per day, administered over a long period, did not decrease the high excretion of calcium in the urine of a diabetic man, and that sodium bicarbonate given to dogs was without effect upon calcium excretion.

The detrimental effects of acid-forming diets upon calcium retention have been noted in several feeding experiments upon animals. Steenbock, Nelson, and Hart (1), Chalmers Watson, and Forbes (2) all agree that decalcification of the bones resulted after prolonged periods on acid-forming rations. The above experiments confirm in general the evidence of an increased calcium excretion resulting from acid-forming diets noted in the present investigations.

CONCLUSIONS.

1. *The base-forming diets consumed in Period II resulted, in every case, in a decided diminution in urinary calcium, while the acid-forming diets of Period IV caused a marked increase in urinary calcium* in three out of four subjects. The amounts of calcium excreted in the urine on the acid-forming diets were from two to three times greater than the amounts excreted on the base-forming diets in every instance.

2. The precentage of total calcium output eliminated through the urine was noticeably decreased on the base-forming diets and increased on the acid-forming diets, while the percentage of

calcium excreted by way of the feces was increased on the base-forming diets and decreased on the acid-forming diets, *showing the tendency of the base-forming diets to divert calcium from the urine to the feces, and of the acid-forming diets to increase urinary calcium at the expense of fecal calcium.*

3. *Three out of the four subjects showed a noticeable increase in total calcium excretion and two showed increased negative calcium balances, not to be accounted for by calcium deficiency in the diets, during the period in which the acid-forming diets were consumed.* It is impossible to draw conclusions as to the effect of the acid-forming diet upon the fourth subject, due to digestive disturbances which arose during the latter part of the experiment.

4. *The total calcium excretion during the period of the base-forming diet was lower than in the preceding period in three subjects,* and about the same in the fourth. *In two instances, the negative calcium balance was diminished on the base-forming diet,* while in two instances, it was increased. In the two latter cases, the increased negative balance can be practically entirely accounted for by the diminished calcium intake of this period. The other two subjects seem to have been able to overcompensate for the decrease in calcium intake by a decrease in calcium excretion.

5. Since the calcium intake furnished by the base-forming diet was appreciably lower than that furnished by either of the other diets, it is impossible to draw definite conclusions as to the influence of the acid-base content of the diets upon calcium retention. However, *the facts stated above tend to indicate that calcium is retained somewhat more readily on a basic diet than on balanced or acid-forming diets, while calcium excretion is greater on an acid-forming diet than on balanced or base-forming diets.*

BIBLIOGRAPHY.

1. Steenbock, H., Nelson, V. E., and Hart, E. B., *J. Biol. Chem.*, 1914, xix, 399.
2. Forbes, E. B., *Ohio Agric. Exp. Station, Bull. 207*, 1919.
3. Sherman, H. C., *J. Biol. Chem.*, 1920, xliv, 21.
4. Sato, A., *Am. J. Dis. Child.*, 1918, xvi, 293.
5. Stehle, R. L., and McCarty, A. C., *J. Biol. Chem.*, 1921, xlvii, 315.
6. Stehle, R. L., *J. Biol. Chem.*, 1917, xxxi, 461.
7. Givens, M. H., and Mendel, L. B., *J. Biol. Chem.*, 1917, xxxi, 421.

STUDIES IN INORGANIC METABOLISM.

III. THE INFLUENCE OF YEAST AND BUTTER FAT UPON CALCIUM ASSIMILATION.

By L. JEAN BOGERT and RUTH K. TRAIL.

(From the Department of Food Economics and Nutrition, Kansas State Agricultural College, Manhattan.)

Because of the importance of calcium assimilation in human beings, especially in such conditions as growth, pregnancy, and rickets, an understanding of various factors which may affect calcium retention is essential. Very few experiments have been carried out to observe the influence of different factors on calcium metabolism in man. The following experiments were undertaken to investigate the possible relation between the vitamine content of the diet and calcium excretion in normal women.

In reviewing the literature on calcium metabolism, many cases of poor utilization of calcium by animals are found. Negative calcium balances have been reported repeatedly, where the calcium content of the diet was known to have been adequate or even abundant. As early as 1908 Hart, McCollum, and Humphrey (1) made observations on a lactating cow, which showed a negative calcium balance persisting throughout an experiment of 110 days. A deficient calcium intake was accompanied by a considerable output of this element in the feces. They believed the skeletal tissues to be ready sources of calcium supply. Hart, Steenbock, and Humphrey (2) later observed disaster in reproduction with cows fed over long periods on a calcium-low ration. Addition of vitamine A (butter fat), of casein, or of both to the diet did not improve the ration for reproduction, while the addition of calcium salts was effective in this respect.

In reviewing his work on high milking cows, supposedly receiving ample amounts of calcium, Forbes (3, 4) pointed out the frequent occurrence of negative calcium balances and the fact that the ad-

dition of easily soluble calcium to the diet did not lead to positive balances.   Meigs, Blatherwick, and Cary (5) found continued negative balances in dry, pregnant cows on a calcium-rich ration of dry alfalfa hay, corn silage, and grain mixture.   They attributed this to nervous disturbances incident upon the experimental procedure.

After the addition of calcium carbonate or phosphate to grain rations fed to a growing pig, Hart, Steenbock, and Fuller (6) found increased retention of both calcium and phosphorus with a resulting heavier skeleton.   In experimenting on growing swine, Hart, Miller, and McCollum (7) noted nutritional failure resulting from a diet of grains.   Both alfalfa, which added inorganic constituents as well as vitamine A, and meat scraps, which supplied more efficient proteins, vitamine A, calcium, and phosphorus, proved good supplements to the grain diet.

In 1913 Steenbock and Hart (8) noticed a favorable effect upon calcium balances in goats after changing from a diet of dry roughage to green pasture.   In a further study (9) made upon both dry and milking goats in 1921, they found similar results on changing from a diet of dry oat straw to fresh green oats.   They also observed that cod liver oil added to the oat straw diet, consistently changed negative calcium balances to positive balances, while orange juice and raw cabbage had no such effect.   It would seem from this work that the factor affecting calcium assimilation and resident in green oats and grasses is present also in cod liver oil.

Robb (10) has cited experiments with guinea pigs, in which the elimination of calcium was twice as great on a diet of dried plants as on a diet of fresh green plants, to which calcium-free orange juice had been added.

Furthermore, the possible relation of vitamine A to calcium retention has been suggested by several workers on rickets.   Shipley, Park, McCollum, Simmonds, and Parsons (11), and Park and Howland (12) studied the beneficial effect of cod liver oil on rats suffering from experimental rickets.   The therapeutic effect of cod liver oil in the treatment of rachitic children has long been recognized. Some substance or substances present in the oil favor calcium deposition in the bones.   Hess does not believe this antirachitic factor to be synonymous with vitamine A (13,14), since, in his experiments, milk did not prevent the development of rickets in children.

He also reported (15) failure of rats to develop rickets on a diet deficient in vitamine A. Lack of active development of bone resulted, however, in these cases and keratitis was also frequent but occurred less often in rats receiving orange juice as a supplement to the diet. McCollum and his collaborators (16,17) found that butter fat did not exert as favorable an influence on bone development in rats as did cod liver oil. He believes the substance which promotes bone growth to be a fourth vitamine (18). It has been pointed out by Howland and Kramer (19) that calcium in the serum of rachitic children may or may not be reduced, but that the phosphorus content of the serum, which is uniformly reduced in rickets, is brought back to normal by administration of cod liver oil.

<div align="center">EXPERIMENTAL.</div>

Four normal women, one college student and three teachers, served as subjects for this experiment. The weights of the subjects ranged from 53.5 to 64.4 kilos and the ages from 21 to 33 years.

The experimental period of 16 days was divided into four 4 day periods, with fore and after periods on calcium-low diet, as described in the preceding papers. The diets of the first and third periods were the same. They consisted of lean beef, rice, skimmed milk, white bread, sugar, corn-starch, and purified fat from nut margarine. These foods were selected to give a diet very low in vitamines. Skimmed milk powder was used, since its vitamine content would be lower than that of fresh, whole milk. The diluted milk powder was also subjected to high temperatures by cooking with rice in a pressure cooker (25 minutes at 10 to 15 pounds pressure). In the second period, yeast was added to the diet to supply vitamine B; and in the fourth period an equal weight of butter fat was substituted for the vegetable fat to give vitamine A. Both the nut margarine and butter were purified by melting at a temperature not exceeding 45°C. and siphoning off the pure fat, which was then filtered. The yeast, added to the diet during Period II, was taken in emulsion with cold water immediately before each meal. Six cakes of compressed yeast were ingested by each subject daily, two cakes per meal.

The diets were planned to meet the energy, protein, and calcium requirements of the subjects. With the exception of the second

period, the amounts of energy, protein, and calcium supplied by the diet were uniform throughout the experiment (Table II). The yeast added to the diet during the second period very slightly increased the intake of calcium, protein, and energy in this period.

TABLE I.

Calcium Content of Foods.

Food.	Calcium.
	per cent
Lean beef..	0.0078
Skimmed milk..	0.107
Bread..	0.041
Rice...	0.009
Yeast..	0.028

TABLE II.

Daily Food Intake.

Food.	Subjects I and J.			Subjects K and L.		
	Periods I and III.	Period II.	Period IV.	Periods I and III.	Period II.	Period IV.
	gm.	gm.	gm.	gm.	gm.	gm.
Lean beef........................	200	200	200	200	200	200
Skimmed milk....................	250	250	250	200	200	200
Vegetable fat....................	72	72		50	50	
Bread...........................	250	250	250	250	250	250
Rice............................	100	100	100	100	100	100
Sugar...........................	75	75	75	50	50	50
Starch..........................	10	10	10	10	10	10
Yeast...........................		84			84	
Butter fat......................			72			50

The diet for Subjects I and J furnished 2,391 calories, 82 gm. of protein, 92 gm. of fat, and 0.395 gm. of calcium. The yeast taken in Period II added 3.9 gm. of nitrogen and 0.023 gm. of calcium.

The diet for Subjects K and L furnished 2,075 calories, 80 gm. of protein, 70 gm. of fat, and 0.341 gm. of calcium. The yeast taken in Period II added 3.9 gm. of nitrogen and 0.023 gm. of calcium.

The protein was in excess of the allowance of 1 gm. per kilo of body weight, suggested by Sherman.[1] The protein of the diet

[1] Sherman, H. C., Gillette, L. H., and Osterberg, E., *J. Biol. Chem.*, 1920, xli, 97.

was fairly high (about 80 to 85 gm. per day), in order to make the diet more palatable and avoid use of too large quantities of carbohydrate and fat. The fat was kept constant, since a variable intake of fat might affect calcium assimilation.[2] The theoretical calcium requirements of the subjects, estimated from Sherman's figure of 0.45 gm. per 70 kilos of body weight,[3] ranged from 0.34 to 0.41 gm. per day. The diets met the theoretical calcium

TABLE III.

Average Daily Intake and Output of Calcium.

Subject.	Weight.	Period.	Diet.		Intake.	Output.			Balance.
						Feces.	Urine.	Total.	
	kg.				*gm.*	*gm.*	*gm.*	*gm.*	
I	60.9	I	Basal.		0.395	0.384	0.131	0.515	−0.120
		II	"	+ yeast.	0.418	0.285	0.142	0.427	−0.009
		III	"		0.395	0.305	0.146	0.451	−0.056
		IV	"	+ butter fat.	0.395	0.255	0.141	0.396	−0.001
J	64.1	I	Basal.		0.395	0.328	0.102	0.430	−0.035
		II	"	+ yeast.	0.418	0.335	0.111	0.446	−0.028
		III	"		0.395	0.296	0.120	0.416	−0.021
		IV	"	+ butter fat.	0.395	0.255	0.124	0.379	+0.016
K	53.5	I	Basal.		0.341	0.248	0.149	0.397	−0.056
		II	"	+ yeast.	0.365	0.152	0.172	0.324	+0.011
		III	"		0.341	0.303	0.153	0.456	−0.115
		IV	"	+ butter fat.	0.341	0.223	0.178	0.401	−0.060
L	56.3	I	Basal.		0.341	0.195	0.159	0.354	−0.013
		II	"	+ yeast.	0.365	0.189	0.156	0.345	+0.020
		III	"		0.341	0.280	0.145	0.425	−0.084
		IV	"	+ butter fat.	0.341	0.163	0.191	0.354	−0.013

requirements in all cases except Subjects J and L, Periods I, III, and IV, while in these instances it was lacking in only 0.02 gm.

The detailed composition of the diets used is given in Table II. The calcium content of the foods is given in Table I. The results of the urine and feces analyses will be found in Tables III, IV, and V. For further details as to experimental procedure consult the first paper of this series.[4]

[1] Givens, M. H., *J. Biol. Chem.*, 1917, xxxi, 441.

[3] Sherman, H. C., *J. Biol. Chem.*, 1920, xliv, 21.

[4] Bogert, L. J., and McKittrick, E. J., *J. Biol. Chem.*, 1922, liv, 363.

DISCUSSION.

The four subjects maintained weight and remained in apparent health during the experimental period. Subject K experienced difficulty with constipation but this condition was relieved by the administration of mineral oil (Nujol) during the first period. The division of the feces was difficult with this subject, as the marker appeared later than with others and persisted for several days. No difficulty was experienced in consuming the diet except

TABLE IV.

Percentages of Daily Calcium Intake Excreted.

Subject.	Period.	Feces.	Urine.	Total.
		per cent	per cent	per cent
I	I	97.2	33.1	130.3
	II	68.1	34 0	102.1
	III	77.2	36.9	114.1
	IV	64.5	35.7	100.2
J	I	83.0	25.8	108.8
	II	80 1	26.5	106.6
	III	74.9	30.4	105.3
	IV	64.5	31.4	95.9
K	I	72.7	43.7	116.4
	I1	41.7	47.1	88.8
	III	88.8	44.9	133.7
	IV	65.4	52.2	117.6
L	I	57.2	46.6	103.8
	II	51 8	42.7	94.5
	III	82 1	42.5	124.6
	IV	47.8	56.0	103.8

in Period II, when there was a feeling of discomfort and lack of appetite. The menstrual period of three subjects was included but no effect on the calcium metabolism was observed.

The data on average daily calcium excretion and balances are found in Table III. The subjects were all somewhat in negative balance during Periods I and III. The amounts of calcium excreted during both of these periods on the basal diet are not uniform for the same subjects, while there are also variations between the

different subjects during the same period.   For instance, Subjects
I, J, K, and L excreted respectively 130.3, 108.8, 116.4, and 103.8
per cent of the calcium intake during Period I; and 114.1, 105.3,
133.7, and 124.6 per cent during Period III (see Table IV).   The
actual amounts of calcium excreted during these periods range
from 0.354 to 0.515 gm., with an average of 0.430 gm. per day.
Although these figures do not show as much individual variation

TABLE V.

Distribution of Calcium in Excreta.

Subject.	Period	Feces.			Urine.		Total calcium.
		Weight.	Calcium.		Volume.	Calcium.	
		gm.	*per cent*	*gm.*	*cc.*	*gm.*	*gm.*
I	I	68.3	2.25	1.537	2,744	0.522	2.059
	II	63.5	1.79	1.140	2,100	0.567	1.707
	III	64.2	1.90	1.220	2,740	0.586	1.806
	IV	44.2	2.30	1.019	3,093	0.565	1.584
J	I	53.6	2.45	1.313	2,418	0.406	1.719
	II	80.3	1.67	1.341	2,280	0.443	1.784
	III	54.9	2.15	1.183	2,294	0.479	1.662
	IV	48.6	2.09	1.018	2,585	0.497	1.515
K	I	74.8	1.33	0.991	4,017	0.597	1.588
	II	59.7	1.02	0.606	3,053	0.689	1.295
	III	72.3	1.67	1.210	4,435	0.612	1.822
	IV	43.5	2.06	0.894	4,170	0.711	1.605
L	I	34.5	2.26	0.780	2,185	0.635	1.415
	II	47.2	1.60	0.758	2,980	0.625	1.383
	III	81.0	1.39	1.122	2,195	0.578	1.700
	IV	45.8	1.43	0.653	2,225	0.762	1.415

as is often found, it has seemed best, in drawing conclusions
concerning the effects of the yeast and butter fat in Periods II
and IV, to compare the calcium excretion and balance of each
subject with the corresponding figures in the basal period immedi-
ately preceding.   Differences between the balances of Periods I
and III in the same subject might be explained by the influence of
previous diet or after effects of the yeast taken in Period II.
Subject J showed a gradual lowering of the per cent of calcium

excreted during Periods I, II, and III, which might indicate that this subject was slow in adjusting to the low calcium intake.

On the examination of the distribution of calcium in the feces and urine (see Table V), it may be observed that the amounts excreted in the urine were fairly constant for each individual, while variations in total calcium output are to be ascribed to variations in the fecal excretion.   There is, however, quite an appreciable amount of calcium in the urine, ranging from 24 to 54 per cent of the total output.   This is contrary to what might be expected from statements of various investigators.   The proportion in the urine seems larger in Subjects K and L.   With Subject K this high urinary calcium runs parallel with a large volume of urine.

It has been noted that the calcium balances during these two basal periods were negative, although the amounts of calcium furnished by the diet were practically adequate to meet the theoretical requirements.   Either the calcium requirement of these subjects must have been higher than the average theoretical requirement given by Sherman, or else poor utilization of the calcium resulted from some undetermined factor.   This factor is presumably the lack of vitamine in the diet, since the same subjects were able to maintain equilibrium or a positive balance later on the addition of yeast or butter fat to the diet.

During Period II, when yeast was added to the diet, considerable discomfort was experienced.   The bulk of feces was increased and they presented a frothy appearance, due to the generation of large amounts of gas in the alimentary tract.   This change in the physical character of the feces might have influenced absorption.   The breaking up of the feces by bubbles of gas, with the probability of greater surface exposed, might have favored absorption.   On the other hand, due to the increased bulk of the intestinal contents, the number of movements was increased, thus creating conditions less favorable to absorption.   There is, however, no evidence in the data to support the assumption that poor absorption occurred, since the dry weight of the feces was not consistently increased (see Table V), and the calcium excretion was decidedly decreased in this period (Table III).   On examination of the distribution of the calcium output (see Table V), it may be observed that the urinary calcium was not changed materially with Subjects I, J, and L, while with Subject K it was increased.   The decrease in total

calcium excretion is due, therefore, to a lowered fecal output, which is brought about by a decrease in weight or in per cent of calcium present in the feces. Comparison of the per cent of the calcium intake excreted (Table IV) and of the calcium balances (Table III) in Period I with those in Period II gives a picture of the effect of the yeast upon calcium *retention*. There is a noticeable lowering in the per cent of calcium intake excreted during Period II in all but one case. This decrease amounted to 28, 28, and 9 per cent for Subjects I, K, and L respectively, but Subject J showed a decrease of only 2 per cent. The yeast seemed to have little or no effect upon calcium excretion in Subject J, as there was but a slight lowering of the negative balance, which continued in Period III. Subject I showed a decided decrease in negative balance, while Subjects K and L showed change from a negative to a positive balance. Hence the lowered calcium excretion cannot be accounted for by the small amounts of calcium contained in the yeast, but must be due to the influence of some other substance or substances added in the yeast. It is interesting to note that there was an increase in fecal calcium output and a return to distinctly negative balance in Period III after withdrawal of the yeast from the diet.

The results obtained in Period IV, when butter fat was substituted for fat from nut margarine, were very similar to those which followed the addition of yeast to the diet. With Subject I the percentage of calcium intake excreted was 14 per cent lower than that of Period III, Subject J, 9 per cent, Subject K, 16 per cent, and Subject L, 19 per cent (see Table IV). The calcium balance of Subject I was changed from negative to equilibrium, of Subject J from negative to positive, and of Subjects K and L to markedly lowered negative balances (Table III). The changes in amounts of calcium excreted were again due to decreased fecal output, brought about, in this case, through a decided drop in weight of feces (Table V). This may indicate a better coefficient of digestibility for several constituents of the diet. Instead of showing a lowering of the per cent of calcium in the feces, as in Period II, the percentage of calcium was increased in every case but one. The urinary calcium of Subjects I and J remained practically constant, but that of Subjects K and L was materially raised. It is very apparent that there is some factor added with the butter fat, which has been favorable to calcium retention.

There seems to be but little difference between the favorable influence of yeast upon calcium retention and that of butter fat, when average daily calcium balances for Periods II and IV are compared with those for Periods I and III.   The average daily balances of the four subjects follow:

Ca
mg.

Period	I	−0.056
"	II	+0.006
"	III	−0.069
"	IV	−0.014

When the balances of only three subjects are considered, since difficulty in marking off the feces into periods may make the results obtained with Subject K less reliable, the results appear as follows:

Ca
mg.

Period	I	−0.056
"	II	−0.005
"	III	−0.053
"	IV	±0.000

When four subjects are considered the yeast seems to have been very slightly more efficient in promoting calcium retention than the butter fat, but when considering only three subjects, there is practically no difference.   It seems surprising that the addition of small amounts of yeast and butter fat to the diet should show such pronounced effects upon calcium assimilation when the experimental periods were limited to 4 days.

CONCLUSIONS.

1. *The addition of yeast to a basal diet, practically free from vitamines, led to lowered excretion of calcium in normal women.* This was shown in three of the four subjects, by the excretion of a smaller percentage of the calcium intake, by a diminished percentage of calcium in the feces, and by decided changes in calcium balance. The negative balance was changed to positive in two cases, while the third subject was brought practically into equilibrium in this period.   The ingestion of yeast had little effect upon the calcium excretion of the fourth subject.

2. *The substitution of an equal weight of purified butter fat for purified fat from nut margarine in the "vitamine-free" diet also*

resulted in decreased calcium elimination. All four subjects excreted a smaller percentage of the calcium intake during this period, and showed a marked lowering of the negative calcium balances in two cases, a change from negative to positive balance in a third, and to equilibrium in a fourth case. The decreased fecal output was brought about by a diminished weight of feces rather than by a lowering of the percentage of calcium in the feces.

3. The above facts might indicate either an improved absorption or a decreased elimination of calcium through the intestinal wall during the periods when yeast or butter fat was taken, and they *suggest some influence of the vitamine content of the diet upon calcium assimilation.*

BIBLIOGRAPHY.

1. Hart. E. B., McCollum, E. V., and Humphrey, G. C., *Wisconsin Exp. Station, Research Bull. 5,* 1908.
2. Hart, E. B., Steenbock, H., and Humphrey, G. C., *Wisconsin Exp. Station, Research Bull. 49.* 1920.
3. Forbes. E. B., Halverson. J. O., and Morgan, L. E., *Ohio Agric. Exp. Station, Bull. 330,* 1918.
4. Forbes, E. B., Schulz, J. A., Hunt. C. H., Winter, A. R., and Remler, R. F., *J. Biol. Chem.,* 1922. lii. 281.
5. Meigs. E. B., Blatherwick, N. R., and Cary, C. A., *J. Biol. Chem.,* 1919, xl, 469.
6. Hart, E. B., Steenbock. H., and Fuller, J. G., *Wisconsin Exp. Station, Research Bull. 30,* 1914.
7. Hart, E. B., Miller, W. S., and McCollum, E. V., *J. Biol. Chem.,* 1916, xxv. 239.
8. Steenbock, H., and Hart. E. B., *J. Biol. Chem.,* 1913, xiv, 59.
9. Hart, E. B., Steenbock. H., and Hoppert, C. A., *J. Biol. Chem.,* 1921, xlviii, 33.
10. Robb, E. F., *Science,* 1920, lii, 510.
11. Shipley, P. G., Park. E. A., McCollum, E. V., Simmonds, N., and Parsons, H. T., *J. Biol. Chem.,* 1920–21, xlv, 343.
12. Park, E. A., and Howland, J., *Bull. Johns Hopkins Hosp.,* 1921, xxxii, 341.
13. Hess, A. F., *J. Am. Med. Assn.,* 1921, lxxvi. 693.
14. Hess, A. F., and Unger, L. J., *J. Am. Med. Assn.,* 1920, lxxiv. 217.
15. Hess, A. F., McCann, G. F., and Pappenheimer, A. M., *J. Biol. Chem.,* 1921, xlvii, 395.
16. McCollum, E. V., Simmonds, N., Shipley, P. G., and Park, E. A., *J. Biol. Chem.,* 1922, l, 5.
17. Shipley, P. G., Park, E. A., McCollum, E. V., and Simmonds, N., *Am. J. Hyg.,* 1921, i. 512.
18. McCollum, E. V., Simmonds. N., Becker, J. E., and Shipley, P. G., *Bull. Johns Hopkins Hosp.,* 1922, xxxiii, 229.
19. Howland, J., and Kramer, B., *Am. J. Dis. Child.,* 1921, xxii, 105.

ANTIKETOGENESIS.

IV. THE KETOGENIC-ANTIKETOGENIC BALANCE IN MAN AND ITS SIGNIFICANCE IN DIABETES.*

By PHILIP A. SHAFFER.

(From the Laboratory of Biological Chemistry, Washington University School of Medicine, St. Louis.)

The first paper in this series (1) describes how, in a search for an explanation of the manner by which the metabolism of carbohydrate prevents the appearance of the "acetone bodies," it was discovered that when glucose is oxidized by hydrogen peroxide in alkaline solution, acetoacetic acid, if present, is also oxidized quite rapidly, whereas in the absence of glucose, acetoacetic acid under otherwise the same conditions is rather resistant to oxidation. An oxidation product of glucose combines with acetoacetic acid and the compound is further oxidized. This "ketolytic" behavior of glucose *in vitro* is evidently analogous to the "antiketogenic" property of carbohydrate in preventing or abolishing ketonuria in the human subject. The reaction has since been studied in greater detail and will be reported on in forthcoming papers.

In a second paper (2) the hypothesis was developed that antiketogenesis in the human subject is based upon a similar ketolytic reaction in the body between acetoacetic acid, the first formed of the "acetone bodies," and a derivative of glucose (or of other antiketogenic substances), the compound being further oxidized; but that failing to react with ketolytic substance acetoacetic acid is resistant to oxidation, accumulates, and (after conversion in part into acetone and hydroxybutyric acid) is excreted. Ac-

---

* The subject matter of this paper formed the basis of a communication before the American Society of Biological Chemists in December, 1921. The methods of calculations then used have been slightly modified and some data have been added, but the conclusions are the same.

cording to this idea, abnormal amounts of acetone bodies would appear among the metabolic products only when the rate of ketogenic catabolism exceeds the rate of antiketogenic (ketolytic) catabolism.

The testing of this hypothesis required the evaluation in common terms of all of the respective ketogenic and antiketogenic influences of protein, fat, and carbohydrate, which substances and their products, in very variable quantities, together make up the "metabolic mixture." The values assigned to these several factors were calculated from theoretical data on the assumptions that each molecule of fatty acid and of the ketogenic amino-acids is the precursor of 1 molecule of acetoacetic acid; that 2 molecules of glycerol (of fat) are converted into 1 of glucose; that the protein amino-acids are converted into glucose to the extent of 3.6 gm. for each gram of (urine) nitrogen; and that carbohydrate, including the glucose from glycerol and protein, exerts its ketolytic action in the form of the six carbon molecule of glucose.

On the basis of these assumptions the data from various subjects were analyzed for the purpose of determining what ratio of ketogenic molecules to antiketogenic molecules (as glucose) exists in the metabolic mixture when small but distinctly abnormal amounts of the acetone bodies first make their appearance, that is, the border-line of ketosis. The results of such calculations appeared to show that the ratio in all of the subjects was approximately 1:1 (the ketogenic factors being calculated as gram mols of acetoacetic acid and the antiketogenic factors as gram molecules of glucose). The same conclusion was also reached from a study (3) of the ketogenic ratio by means of respiratory quotients; and Woodyatt (4) has found that such a conclusion is in accord with his clinical experience. Hubbard and Wright (5) have reported experiments on the same topic, and their data are quite in harmony with the same general conclusion.

The fact that with a considerable number of different subjects, worked upon by different observers, one finds at the threshold of ketosis an approximately constant ratio between the number of molecules of the precursors of acetoacetic acid and of glucose in the metabolic mixture, must mean that the further oxidation of acetoacetic acid constantly taking place under normal conditions is accomplished through a chemical reaction with a derivative

of glucose, and presumably this reaction is more or less similar to, if not the same as, the *in vitro* ketolytic reaction.

If this is the case and if one can evaluate the various factors in the ketolytic reaction with sufficient accuracy it should be possible to draw up a ketogenic-antiketogenic balance for any given subject and from the excess of ketogenic molecules, predict the amount of acetoacetic acid (total "acetone bodies") which would be excreted. The ability thus to predict the effect of any ketogenic balance would be of great scientific interest in connection with the intermediary metabolism of the three foodstuffs, and besides would have a very considerable practical value as the basis for the dietetic treatment of diabetes and obesity. In diabetes especially, such information would permit a rational formulation of diets to accomplish the essential aims, (*a*) to spare the carbohydrate tolerance from overstrain and (*b*) to avoid by safe margin ketosis and its consequences, while maintaining the plane of nutrition as high as permissible. The diabetic dietary has indeed already been considered from the point of view of the ketogenic balance (4 to 8) and a development along these lines appears full of promise.

The evaluation of the factors of the ketogenic balance is obviously a complex and difficult problem, not only because of the uncertainty as to the ketogenic *versus* antiketogenic equivalence of fat, proteins, and carbohydrate, but also for the reason that any chemical reaction taking place in such a many phase system as the animal body doubtless proceeds under different conditions in different tissues or cells and at different times and is influenced by many unknown factors. Furthermore, the calculation of an approximately correct ketogenic balance sheet requires an accurate knowledge of how much protein, sugar, and fat are being catabolized and this information is in nearly all cases uncertain,—especially uncertain regarding fat. And since all of the various errors in the determinations and assumptions underlying the calculation of the ketogenic balance are accumulated in the end-result, the "balance," it can scarcely be expected that we may immediately arrive at even an approximate estimate of the quantities involved in the ketogenic balance. The attempts to calculate a "balance" or absolute excess of ketogenic or antiketogenic factors puts the hypothesis to a very severe test, so severe that perhaps uniform success would not be expected. Neverthe-

less, the data to be presented indicate that an approach to this complex problem is possible and that by justifiable assumptions, based upon the general hypothesis as to the ketolytic reaction, one may explain in many cases not only the threshold of ketosis, but also the amounts of total acetone bodies excreted by different human subjects with marked ketosis. The hypothesis thus appears to gain strong support, and may be applied with some confidence to certain fundamental problems in the metabolism of diabetes.

In a former paper (2) it was pointed out (pp. 466 and 471) that although a molecular ratio of about 1:1 appears to exist at the threshold of ketosis, the amount of acetone bodies actually excreted by subjects with marked ketosis is much less than corresponds to the calculated excess of ketogenic over total glucose molecules; and it was there suggested that this fact indicates error in some of the assumptions or some of the factors in the calculation. An example will illustrate the discrepancy. Benedict's fasting subject "L" (9) on the 14th day of fast, catabolized protein corresponding to 10.43 gm. of urine nitrogen and 117 gm. of body fat, but oxidized no carbohydrate. Such a metabolic mixture would represent, according to the first method of calculation (2, p. 457) the following:

	Ketogenic.	Antiketogenic.
	millimols	*millimols*
10.43 gm. N.....................	104 (10.43 × 10)	208 (10.43 × 20)
117 gm. fat.....................	401 (117 × 3.43)	67 (117 × 0.57)

505−275 = 230 excess ketogenic millimols × 0.104 = 24 gm. total hydroxybutyric acid expected.

The actual excretion was not more than about 7 gm. of total hydroxybutyric acid, or about 17 gm. less than calculated. Such discrepancies appear to be regularly found with marked ketosis, and probably indicate one of the following possibilities: (*a*) Some of the accumulating acetoacetic or hydroxybutyric acids are oxidized without reaction with the ketolytic substances; (*b*) the estimates of the amounts of acetoacetic acid derivable from fat and protein are too high; (*c*) glucose, or other ketolytic substances derived from protein and glycerol of fat have greater antiketogenic influence than the values assigned.

Of these three possibilities, the first, the oxidation of keto- or hydroxy-acid without ketolytic reaction cannot be decided at present, and may perhaps be held in reserve as a sort of last resort in case the facts cannot be otherwise interpreted. To admit at the outset such a variable would greatly complicate the calculations needed to test the other two possibilities, and for the present it is tentatively assumed that no such direct oxidation takes place.

Since for reasons already given (2, p. 467), it seemed unlikely that the values adopted for the ketogenic factors were very greatly in error, it appeared necessary first to question the values for the antiketogenic factors. Antiketogenic substances are derived from certain of the amino-acids, and probably from glycerol of fat as well as from carbohydrate. Whether the active ketolytic substances from all three sources are different, or are the same cannot be definitely settled at present, but our original assumption seems the more probable. Hubbard and Wright (5) interpret their data in support of this assumption. The fact that the blood of fasting normal subjects continues to contain the usual amount of sugar, indicates its continued formation from substances other than carbohydrate, in normal (as well as diabetic) individuals, and supports the idea that glucose is a normal intermediate in protein (and glycerol?) metabolism.

We shall, therefore, suppose that the antiketogenic action of protein and of fat is represented by the amounts of glucose to which they may give rise, and consider the maximum ketolytic effect of glucose.

The first possibility that suggested itself was that 1 molecule of glucose (or more strictly its active ketolytic derivative) may react with 2 instead of with 1 molecule of acetoacetic acid. This idea seems very probable from the fact that further work on the *in vitro* ketolytic reaction shows that under proper conditions 1 molecule of glucose accomplishes the disappearance of 2 molecules of keto-acid, when the latter is present in excess. Other related substances, such as other hexoses, glycol aldehyde and glyoxal, behave similarly, with the latter no oxidation being necessary. A detailed account of the *in vitro* reaction will be presented in later papers and need not be entered upon here except to state that the evidence points to a reaction between 1 molecule of a

glucose derivative (probably glucosone), and 2 of acetoacetic acid.
If 1 molecule of glucose (or its derivative) reacts with 2 of keto-
acid, the effect would be to double the values assigned the anti-
ketogenic factors in our calculations.

When this modification is tried on various data, the discrepancy
is usually much less, *but it leads to the expectation of considerably
less acetone bodies than the amounts actually excreted.* Using the
data of Subject "L" above mentioned as an example, the antiketo-
genic sum would be $275 \times 2 = 550$ antiketogenic equivalents
and would *exceed* by 45 the sum of ketogenic millimols, and if the
reaction in the body as a whole were strictly quantitative, no
ketosis would be expected.

Of course such a reaction cannot be *strictly* quantitative in the
body as a whole, but with marked ketosis it may approach a
quantitative relationship. When the metabolic mixture is such
that a large excess of keto-acid molecules is formed compared
with the total glucose being burned, one may imagine that prac-
tically every glucose molecule encounters keto-acid, with conse-
quent approach to complete utilization of all available antiketo-
genic substance. The best that can be done is to choose subjects
with marked ketosis, and tentatively assuming that the ketolytic
reaction in the body as a whole is approximately *quantitative under
such conditions*, determine by trial what values for the ketogenic
and antiketogenic effects of protein and fat best correspond
with the amounts of total acetone bodies actually excreted.

Since we feel confident from the *in vitro* work that the ketolytic
reaction ratio is 1 of glucose to 2 of keto-acid, we interpret the
discrepancy in the calculated "balance" (above illustrated) to
mean either that the amount of keto-acid derived from protein
is larger than indicated by the analyses of leucine, tyrosine, and
phenylalanine, or that the amount of glucose derived from pro-
tein and glycerol is less than the values used in the calculations.

The latter alternative first seemed the more likely for the follow-
ing reason. The value adopted in earlier papers for the glucose
equivalent of protein was based upon the maximum D:N ratio
of 3.6:1, which appears to have been established by the work of
Lusk and others. At the time of writing the earlier papers, the
fact was overlooked that since this ratio actually is an expression
of the relative amounts of *urinary* nitrogen and glucose, and since

it is assumed that all glucose formed in the body is excreted when the D:N ratio is 3.65:1, one is hardly justified in accepting that ratio as indicating the amount of glucose derivable from protein, *and* assuming an *additional* derivation from glycerol. For if the latter assumption is correct, some portion of the glucose excreted by the "total" diabetic or the completely phlorhizinized animal must have come from glycerol, and the D:N ratio would therefore be greater than actually represents the maximum derivation from protein. The assumption as to glycerol thus seemed inconsistent with the value adopted for the antiketogenic fraction of protein. Based on this reasoning, I adopted a lower value for the glucose equivalent of protein, corresponding to a D:N

TABLE 1.

Ketogenic-Antiketogenic Influence of Foods.

Basis of calculations presented to American Society of Biological Chemists, and since slightly revised.

	Per gram.					Per calorie.			
				Excess.				Excess.	
	Calories.	Keto-genic.	Anti-keto-genic.	Keto-genic.	Anti-keto-genic.	Keto-genic.	Anti-keto-genic.	Keto-genic.	Anti-keto-genic.
	milli-mols	*milli-mols*	*milli-mols*	*milli-mols*	*milli-mols*	*milli-mols*	*milli-mols*	*milli-mols*	*milli-mols*
Fat..................	9.46	3.43	1.14	2.29		0.363	0.1205	0.242	
Protein, 1 gm. N	26.5	11.7	33.0		21.3	0.442	1.245		0.803
Carbohydrate glucose.	3.76	0	11.12		11.12		2.96		2.96

ratio of 3:1, (1 gm. of urine $N \times 16.5$ = millimols glucose from protein). Using this value instead of $N \times 20$, the value used in earlier papers and multiplying the total mols of glucose by 2 to give its antiketogenic equivalents, I analyzed data from a number of subjects with rather striking agreement between the calculated and actual hydroxybutyric acid (total excretion). These results and certain deductions from them were presented before the American Society of Biological Chemists in December, 1921 (10). The factors used in those calculations are given in Table I. These values very often work surprisingly well, but since they were later modified it is hardly worth while to print the tables of calculations.

Later analyses of other data have made me doubt the wisdom of reducing the estimate of the glucose equivalent of protein, and point rather to the conclusion that the first estimate of the *keto-genic* value of protein is too low. The reasons for this doubt are briefly as follows. Well authenticated results from a very few notably severe cases of diabetes show on occasional periods an excretion of glucose definitely greater than corresponds to a $D:N$ ratio of 3.6 and which can be accounted for best by assuming the formation of about 3.6 gm. from protein for each gram of nitrogen *and* the conversion of glycerol into glucose. The results for the Du Bois and Lusk case "Cyril K" (11) on December 15 to 17 are a good example; 57 gm. of glucose were excreted during 3 days over and above the amount calculated from a maximum $D:N$ of 3.65. The explanation seems justifiable that the surplus glucose was formed from the glycerol of fat burned. If such be accepted for a single case, it would seem necessary to assume as great a derivation of glucose from protein and glycerol by other subjects who, however, burn all or some portion of it. But if glucose is formed to this extent, the estimate of the *ketogenic* value of protein must be *increased*, because the result of the metabolism of protein is, in many subjects at any rate, the excretion of more hydroxybutyric acid than would otherwise be expected from the calculated "balance." Since the analyses of muscle protein for the ketogenic amino-acids probably represent minimum values, a higher ketogenic value than that calculated would not be surprising.

After many trials on the data of many subjects, I have, therefore arbitrarily increased the protein ketogenic factor by 50 per cent. The justification for the change is that calculations show better agreement.

The factors for the calculation of the ketogenic balance, modified as explained above, may be summarized as follows. In this form there are only two changes from the factors used in the earlier papers; an increase in the ketogenic value of protein, and the multiplication of the total mols of glucose by 2 to give the "total antiketogenic equivalents."

Ketogenic Equivalent as Millimols Acetoacetic Acid.

Protein   (*a*) **gm. urine N** $\times$ **15**

Fat   (*b*) gm. fat burned $\times \dfrac{3 \times 1,000}{874}$ = **gm. fat** $\times$ **3.43**

or, since 1 gm. fat = 9.46 Cal.,

Calories of fat burned $\times \dfrac{1}{9.46\,\text{Cal.}} \times 3.43$ = **fat Cal.** $\times$ **0.363**

Glucose Equivalents in Millimols.

Carbohydrate   (*c*) gm. glucose $\times \dfrac{1,000}{180}$ = **gm. glucose** $\times$ **5.56**

Protein   (*d*) gm. urine N $\times \dfrac{3.6 \times 1,000}{180}$ = **urine N** $\times$ **20**

Fat   (*e*) gm. fat burned $\times \dfrac{1 \times 1,000}{874 \times 2}$ = **gm. fat** $\times$ **0.57**

or Calories of fat burned $\times \dfrac{1 \times 1,000}{874 \times 2 \times 9.46}$ = **Cal. of fat** $\times$ **0.06**

"Ketogenic ratio" $\dfrac{\text{Total ketogenic mols}}{\text{Total mols glucose}} = \dfrac{a + b}{c + d + e}$

"Ketogenic balance" = Total ketogenic mols $-$ (2 $\times$ total glucose mols)

$$= (a + b) - 2\,(c + d + e).$$

Testing the Ketogenic-Antiketogenic Factors.

We may now see how the factors developed above work out when applied to data from various subjects with severe ketosis. With the exception of one case of diabetes, all of the data are taken from the work of others already published. A large part of the suitable data which I have found in the literature is included, and I have *not* excluded data which show poor agreement with the calculations.

For the calculation of the ketogenic balance it is necessary to know the amounts of the three foodstuffs metabolized, and to be able to assume that the proportions remained approximately constant during the period. On the latter point one can have no assurance. The protein metabolized is reckoned from the urinary nitrogen. Food carbohydrate, if not excreted as glucose, is sup-

posed to be burned; in the cases with severe ketosis to be discussed,
fasting and diabetics, very little glucose (except from protein and
glycerol) could have been burned, and the assumption is approximately
correct.   The only way to determine the amount of fat
burned is by calculation from the total metabolism, and this is
one of the chief errors and difficulties.   Few if any subjects with
marked ketosis, and with determinations of the total acetone,
have been studied for whole days or long periods in a respiration
apparatus.   Many data are available which include determinations
of resting metabolism for short periods, but considerable
additions must be added to cover muscular activity and other increases
of metabolism throughout the day, and the amount of
these additions is scarcely better than a guess.   Until suitable
subjects are studied continuously in a calorimeter, all one can do
is to estimate the total calories of metabolism.   From this doubtful
estimate the fat burned may be calculated by subtracting the
protein calories (urine $N \times 26.5$) and carbohydrate if any (glucose
$\times 3.76$), and dividing the remainder by 9.4 Cal.

In most of the cases to be analyzed the data regarding total
metabolism, and therefore of fat burned, are admittedly questionable,
and to any who are inclined to doubt their value on this account,
it can only be said that they represent the best now available

Fasting Subjects.

Subject "L" during 31 Day Fast (F. G. Benedict, 9).—The data
from this well known experiment are among those most suitable
for our calculation, and have been used on earlier pages.   For each
of the 31 days of the fast, Benedict records the probable total
energy exchange for 24 hours.   The respiratory quotients on the
4th day indicate that only 4.3 gm. of glycogen were oxidized,
and we have assumed that beginning with the 5th day no carbohydrate
(other than that derived from protein and fat) was burned.
From the 5th day on, the total energy was thus derived from fat
and protein, and the total calories minus the protein calories
(urine $N \times 26.5$) equals the fat calories.   The ketogenic balance
is calculated by multiplying the corresponding amounts of protein
(nitrogen) and of fat (or calories from fat) by the respective factors
given on page 407.   The excess of ketogenic over antiketogenic
equivalents multiplied by 0.104 (104 being the molecular weight

of hydroxybutyric acid), gives the *expected total* hydroxybutyric acid in grams. Unfortunately for our purpose, the acetone and acetoacetic acid excretion was not determined and Benedict records only the urinary hydroxybutyric acid; the *total* acetone bodies, however, include not only acetone and acetoacetic acid of urine but acetone exhaled, and this total we have estimated at about 50 per cent greater than the amount of the recorded hydroxybutyric acid. While such an estimate is admittedly a rough approximation, the total is not large and the error cannot be very serious.

The details are given in Table II. It will be noted that although there are fluctuations in the degree of agreement between expected and excreted total hydroxybutyric acid from day to day (Columns 13 and 14) the uniform similarity of the figures is striking. The totals for 27 days are 133 gm. expected and 162 gm. excreted, an agreement which is close enough to indicate that the basis of calculation is at least approximately correct.

Fasting Obese Woman "Mrs. McK" of Means (12) and Folin and Denis (13).—This subject underwent three fasts of 4 and 5 days each, separated by an interval of several days with food. Toward the close of each fast she excreted from 17 to 24 gm. of total hydroxybutyric acid. The resting metabolism was determined by Means from which the total energy exchange may be roughly estimated by adding 10 per cent to allow for the increased metabolism resulting from muscular activity. This addition is admittedly subject to considerable error, but is approximately the estimate by Du Bois to cover the extra metabolism from muscular activity of a hospital patient, and is perhaps as close as it is possible to estimate the daily energy exchange of such subjects without continuous determinations.

To the energy exchange it is theoretically necessary to add the calorie value of the material excreted unburned, in this case the acetone bodies, since they were derived from fat and protein catabolized, even though incompletely. The sum of these quantities then represents the calorie value of the total material catabolized, which is assumed to consist in this case of fat and protein. And the total calories minus the calories from protein equals the calorie equivalent of the fat burned.

TABLE II.

Ketogenic Balance of Benedict's Subject "L" during Fast.

Days of fast. (1)	Total calories. (2)	Urine N. (3)	Calories from fat. (4)	Ketogenic millimols.			Glucose equivalent millimols.			Antiketogenic equivalents. (11)	Excess ketogenic millimols. (12)	Total hydroxybutyric acid	
				Fat. (5)	Protein. (6)	Total (7)	Fat. (8)	Protein. (9)	Total. (10)			Expected. (13)	Excreted. (14)
												gm.	gm.
5	1,609	10.41	1,269	460	156	616	76	208	284	568	48	5.0	3.2
6	1,537	10.18	1,267	460	152	612	76	203	279	558	54	5.6	5.2
7	1,540	9.79	1,280	465	148	613	77	196	273	546	67	7.0	4.2
8	1,503	10.27	1,231	447	154	601	74	205	279	558	43	4.5	2.4
9	1,481	10.74	1,196	434	161	595	72	215	287	574	21	2.2	5.2
10	1,426	10.05	1,160	421	151	572	70	201	271	542	30	3.1	5.2
11	1,385	10.25	1,113	404	154	558	67	205	272	544	14	1.4	2.1
12	1,410	10.13	1,141	414	152	566	68	202	270	540	26	2.7	3.6
13	1,349	10.35	1,075	391	155	546	65	207	272	544	4	0.4	6.3
14	1,394	10.43	1,117	406	157	563	67	208	275	550	13	1.4	7.0
15	1,331	8.46	1,107	402	127	529	66	169	235	470	59	6.1	2.4
16	1,319	9.58	1,065	387	144	531	64	191	255	510	21	2.2	7.8
17	1,300	8.81	1,066	387	132	519	64	176	240	480	39	4.0	5.4
18	1,257	8.27	1,038	377	124	501	62	165	227	454	47	4.9	6.6
19	1,261	8.37	1,039	377	125	502	62	167	229	458	44	4.6	10.5
20	1,252	7.69	1,048	380	115	495	63	154	217	434	61	6.3	6.6
21	1,276	7.93	1,066	387	119	506	64	158	222	444	62	6.4	7.5
22	1,235	7.75	1,030	374	116	490	62	155	217	434	56	5.8	4.6
23	1,230	7.31	1,036	376	110	486	63	146	209	418	68	7.1	9.0
24	1,254	8.15	1,038	377	122	499	62	163	225	450	49	5.1	10 0
25	1,251	7.81	1,044	379	117	496	63	156	219	438	58	6.0	6.6
26	1,246	7.88	1,037	376	118	494	62	157	219	438	56	5.8	9.2
27	1,255	8 07	1,041	378	121	499	63	161	224	448	51	5.3	6.0
28	1,289	7.62	1,087	395	114	509	65	152	217	434	75	7.8	7.4
29	1,247	7.54	1,047	380	113	493	63	151	214	428	65	6.8	8.4
30	1,239	7.83	1,031	374	117	491	62	156	218	436	55	5.7	8.1
31	1,281	6.94	1,097	298	104	502	66	139	205	410	92	9.6	6.7

As an example of the calculation, on the 4th day of the first fast the calorie value of total material metabolized was:

$$110 \text{ per cent} \times 260 \text{ cc. } O_2 \text{ per min.} \times 1,440 \text{ min.} \times \left.\right\} = 1,924 \text{ Cal.}$$
$$4.67 \text{ Cal. per liter } O_2$$

$$24.6 \text{ gm. total hydroxybutyric acid} \times 4.69 \text{ Cal.} = \underline{115 \text{ Cal.}}$$
$$2,039 \text{ Cal.}$$

$$2,039 - (9.4 \text{ gm. N} \times 26.5) = 1,790 \text{ Cal. from fat.}$$

Ketogenic Balance.

	Ketogenic.	Glucose.
	millimols	*millimols*
9.4 gm. N........	141 (9.4 × 15)	188 (9.4 × 20)
1,790 Cal. from fat	649 (1,790 × 0.363)	107 (1,790 × 0.06)
Total.....................	790	295

790 − (2 × 295) = 200 excess ketogenic millimols.

$$200 \times \frac{104}{1,000} = 20.8 \text{ gm. total hydroxybutyric acid expected.}$$

There were actually excreted on this day the equivalent of 24.6 gm. of total hydroxybutyric acid. The details of the same calculation for the last 2 days of each fast are given in Table III, from which it appears that the calculated expectation (Column 16) on the last day of each fast at least approximates the amount excreted (Column 17). On the 4th day of the second fast and the 3rd day of the third fast much less was excreted than expected and this is interpreted to mean that on these days the subject was still using some glycogen. It is not unlikely that glycogen was burned also on the last days of the second and third fasts, and that better agreement might have resulted if the fasts had been prolonged. The "adaptation" to repeated fasts illustrated by this patient, and pointed out by Folin and Denis probably is to be explained by the longer retention of stored glycogen, and is an important phenomenon requiring further investigation. The greater ketosis of this subject is evidently due to the relatively smaller catabolism of protein, about 7 per cent of the total calories compared to about 18 per cent in the case of Subject "L" above.

Grafe's Fasting Subject " M.K." (14).—A woman of 27 years, weight 52 kilos fasted about 3 weeks in a catatonic stupor. During the latter part of the period, the subject was placed in a respiration chamber for many hours daily, and the total metabolism and respiratory quotients carefully determined. From these data, Grafe calculated the total energy exchange for 24 hours. A num-

TABLE III.

Ketogenic Balance, "Mrs. McK," Fasting Obese Women of Means and Folin and Denis.

Fast. (1)	Day of fast. (2)	O₂ per min. (3)	Total calories per 24 hrs. (4)	Urine N. (5)	Calories from protein (5.3 × 26.5) (6)	Calories from fat. (7)	Ketogenic millimols. Fat (calories × 0.363). (8)	Ketogenic millimols. Protein (N × 13). (9)	Ketogenic millimols. Total. (10)	Glucose millimols. Fat (calories × 0 06). (11)	Glucose millimols. Protein (N × 20). (12)	Glucose millimols. Total. (13)	Antiketogenic millimols (13) × 2. (14)	Ketogenic excess millimols (10) − (14). (15)	Total hydroxy-butyric acid. Expected. (16)	Total hydroxy-butyric acid. Excreted. (17)
		cc.		gm.											gm.	gm.
I	3	261	2,027	8.7	231	1,796	652	131	783	108	171	282	564	219	22.8	20.7
	4	260	2,639	9.4	249	1,790	649	141	790	107	188	295	590	200	20.8	21.6
II	4	234	1,770	5.5	146	1,624	590	82	672	98	110	208	416	256	26.6	8.6
	5	232	1,796	5.2	138	1,658	602	78	680	100	104	201	408	272	28.3	17.3
III	3	221	1,651	4.7	125	1,526	554	71	625	92	94	186	372	253	26.3	3.5
	4	225	1,759	4.5	120	1,639	595	68	663	98	90	188	376	287	29.8	20.1

ber of urine analyses were also made, including total nitrogen, acetone + acetoacetic acid, and hydroxybutyric acid, the latter by Magnus-Levy's extraction method. The data given by Grafe are thus almost ideal for our calculation of the ketogenic balance. For the purpose I have taken Grafe's own figures for the calories from fat, and these with the urine total nitrogen, multiplied by the factors stated above (used for all calculations in the present paper) give the data for the ketogenic balance on 5 days, the 15th to 19th of the fast, presented in Table IV. In the last column (Column 15) of the table are found the total amounts of acetone bodies excreted, expressed as grams of hydroxybutyric acid, and in the preceding column (Column 14) the amount expected from the calculated "balance." *The agreement is astonishingly good,* the total calculated for the 5 days being 88 gm. and the amount excreted (exclusive of acetone exhaled) is 72 gm.

Bönniger and Mohr's Fasting Subject Schenk (15).—"Hungerkünstlerin," 48 years old, 152 cm. height, and 54.41 kilos weight (on 2nd day of fast), in bed except for short periods. Data are recorded on the total hydroxybutyric acid (including acetone exhaled) and the total nitrogen excretion. The metabolism was determined and publication of the results by Mohr is promised but has not been found in the literature.

In the absence of better information, we have estimated the metabolism and calculated the ketogenic balance during 4 days, the 5th to 8th day of fast. The average weight during this period was 51.6 kilos, which according to the Du Bois height-weight chart gives a surface area of 1.46 sq. m., and 1.46 × 36.9 cal. × 24 hrs. = 1,293 cal. per day, normal basal for a woman of the same size. Add 10 per cent to cover movements in bed, and assume that the decrease in metabolism as a result of fasting was proportionately the same, as found by Benedict with Subject "L" (average of 12 per cent less on 5th to 8th fast days than on 1 fast day).

1,293 + 129 = 1,422 − 159 = 1,263 cal.

The average nitrogen excretion for the 4 days was 7.25 gm. × 26.5 = 192 cal. from protein. 1,263 − 192 = 1,071 cal. from fat.

TABLE IV.

Ketogenic Balance of Fasting Woman "M. K.," Catatonic Stupor (Grafe).

Date	(1) Day of fast	(2) Length of respiration period. hrs.	(3) Total calories (Grafe).	(4) Calories from fat (Grafe).	(5) Urine N. gm.	Ketogenic millimols.			Glucose equivalent millimols.			(12) Antiketogenic equivalent (11) × 2.	(13) Excess ketogenic milli-mols.	Total hydroxy-butyric acid.	
						(6) Fat (calories × 0.363).	(7) Protein (N × 13).	(8) Total (6) + (7).	(9) Fat (calories × 0.05).	(10) Protein (N × 20.5).	(11) Total (9) + (10).			(14) Calculated. gm.	(15) Excreted. gm.
Oct. 12	15	10.0	1,210	1,101	5.73	400	86	486	66	115	181	362	124	12.9	12.6
" 13	16	4.5	1,325	1,154	5.28	426	79	505	70	105	175	350	155	16.1	17.4
" 14	17	4	1,500	1,371	4.92	498	74	572	82	98	180	360	212	22.0	12.3
" 15	18	11.0	1,370	1,231	4.86	447	73	520	74	97	171	342	178	18.5	16.4
" 16	19	5.0	1,336	1,203	4.55	437	68	505	72	90	162	321	181	18.8	13.7

From these figures the ketogenic balance is as follows:

	Ketogenic.	Glucose equivalents.
	millimols	*millimols*
Protein, 7.25 gm. N........	109 (7.25 × 15)	145 (7.25 × 20)
Fat, 1,071 cal...............	389 (1,071 × 0.363)	64 (1,071 × 0.06)
Total...................	498	209

498 − (209 × 2) = 80 = 8.3 gm. hydroxybutyric acid expected.
Average actually excreted per day = 17.0 gm.

The agreement is not close, but considering the uncertainty as to total metabolism, is moderately satisfactory. If the total calories were 200 or 300 higher per day, almost perfect agreement would result.

Cases of Brugsch (16).—Among the data on ketosis of starvation to be found in the literature, are the observations of Brugsch during the last 10 days of a 30 day fast of Succi, and on an extremely emaciated woman with a complete esophageal stenosis. Succi was found to excrete from 7 to 12 gm. of total hydroxybutyric acid per day, while the urine of the emaciated woman contained no acetone bodies. Since information as to Succi's total metabolism at this time is not available, and because of the doubtful method used for determination of hydroxybutyric acid, we shall not attempt the calculation of his ketogenic balance; the results of such calculation would not be exceptional. But the remarkable absence of ketosis in the emaciated patient merits brief comment. Brugsch interpreted the absence of ketosis as being due to the fact that the body fat had been absolutely depleted, the energy requirement being supplied wholly from protein; and hence he concluded that body fat is the source of the acetone bodies. But it is scarcely possible that even so extremely emaciated a person as this one, burned no fat. The woman was 56 years old, 175 cm. tall, and weighed 32 kilos. Her nitrogen excretion was 5.46 gm. (on the day before operation), corresponding to protein whose maximum available energy value is 5.46 × 26.5 cal. = 145 cal. Her total metabolism is unknown, but was doubtless at least 20 cal. per kilo per day or say 600 cal. Something like 450 cal. or 75 per cent of the total must therefore have been derived from the small (and doubtless invisible) remainder of body fat. From

the metabolism of such a mixture, we should expect the following ketogenic balance.

	Ketogenic.	Antiketogenic equivalent.
	millimols	*millimols*
Protein, 5.46 gm. N........	82 (5.46 × 15)	218 (5.46 × 20 × 2)
Fat, 450 cal...............	163 (450 × 0.363)	54 (450 × 0.06 × 2)
Total...................	245	272

Since the ketogenic millimols are less than the available antiketogenic equivalents, little or no ketosis would be expected. We should say, therefore, that the absence of ketosis was due to relatively high protein metabolism (about 24 per cent of the total) and that the glucose formed from protein was sufficient to supply the necessary ketolytic substance.

Subjects of Wilder and Winter (7).—Very recently these authors have reported valuable data from sixteen different subjects all but three of whom excreted small amounts of total acetone and represent the border-line or threshold of ketosis.   One of the three was an epileptic, Case A366687, on the 11th day of fasting and had sufficiently marked ketosis (about 10 gm. total hydroxybutyric acid) to justify a calculation of the ketogenic balance.   The data given by Wilder and Winter may be used as follows: The determined resting metabolism (1,335 cal. for 24 hours) increased by 10 per cent to allow for muscular activity,[1] gives 1,469 cal. as the estimate of the heat produced.

To this the calorie value of the total hydroxybutyric acid excreted is added, the sum being the estimated value of the total material metabolized.

cal

Resting metabolism (determined for short periods)......... 1,335
Adding for muscular activity (10 per cent)................. 134
Calorie value of 5.7 gm. total acetone × 1.8 = 10.25 gm.
  hydroxybutyric acid × 4.7.............................. 48

Estimated total metabolism............................... 1,517

1,517 cal. − (6.62 gm. urine N × 26.5) = 1,341 cal. from fat.

[1] Since the subject was fasting, the additional allowance of 10 per cent for food made by Wilder and Winter is omitted.

	Ketogenic.	Antiketogenic.
	millimols	*millimols*
Fat..................	487 (1,341 × 0.363)	161 (1,341 × 0.06 × 2)
Protein.............	99 (6.62 × 15)	265 (6.62 × 20 × 2)
Total.............	586	426

Excess = 160 ketogenic millimols $\times \dfrac{104}{1,000}$ = 16.6 gm. calculated total hydroxybutyric acid; excreted 10.25 gm.

The ketosis is thus approximately accounted for. The other two cases with marked ketosis were diabetics on diet and will be mentioned later.

Severe Diabetics with Marked Ketosis.

"*Cyril K*" *of Lusk, Du Bois, and Collaborators (11)*.—The admirably complete data from this subject are exceptionally valuable and permit a reasonably satisfactory analysis of the ketogenic factors, and were so used by the writer in a former paper (2). A recalculation based upon the revised ketogenic-antiketogenic values is presented in Table V. The same method of calculation is followed as with the preceding fasting non-diabetic subjects. The total heat produced for 24 hours is taken as 110 per cent (× 24 hours) of the determined hourly resting heat production (the indirect cal. per hour from Table V of the paper by Lusk and coworkers). To this value is added the calories of total hydroxybutyric acid and of the "extra" glucose[2] excreted, the sum being the caloric equivalent of the total material catabolized (some of it incompletely) during the 24 hours. This consisted only of protein and fat (no food CH was burned) and the value of the latter is, Total calories − protein calories (N × 26.5) = calories of fat catabolized.

From these data the total ketogenic and equivalent antiketogenic millimols are calculated, and from the latter are subtracted the antiketogenic equivalents of the "extra" glucose (from protein or glycerol) which the subject excreted and thus failed to use. The excess of ketogenic over the *net* antiketogenic milli-

[2] By "extra" glucose is meant that derived from protein or fat and excreted. It is assumed to be represented by the excess of urine glucose over the food carbohydrate.

TABLE V.

Ketogenic Balance of Severe Diabetic, "Cyril K.," of Geyhart, Aub, DuBois, and Lusk.

(1) Date	(2) Food CH.	(3) Urine glucose	(4) Extra glucose	(5) Urine N.	(6) Resting calories per hr. observed	(7) 110 per cent of determined calories per day	(8) Caloric value extra glucose + β-hydroxybutyric acid	(9) Total calories of metabolism	(10) Protein calories, N × 26 5	(11) Calories from fat	Ketogenic millimols			Glucose equivalent					(20) Antiketogenic equivalent net glucose × 2	(21) Excess ketogenic millimols	Total β-hydroxy-butyric acid	
											(12) Fat calories × 0.363	(13) Protein N × 15	(14) Total	(15) Fat (calories × 0.06)	(16) Protein N × 20	(17) Gross total	(18) Deduction for extra glucose	(19) Net total			(22) Calculated	(23) Excreted
																					gm.	gm.
Oct. 15	24.0	168	144	36.4	81.9	2,160	916	3,076	965	2,111	766	546	1,312	127	728	855	800	55	110	1,202	125	±80
" 16	0.4	153	153	38.3	76.4	2,020	951	2,971	1,015	1,956	719	575	1,285	118	766	884	851	33	66	1,219	127	±80
" 17	0.4	140	140	36.3	73 2	(2,000)	935	2,935	962	1,973	716	544	1,260	119	726	845	779	66	132	1,128	117	+87
" 18	Fast.	55	55	20.0		1,935	479	2,414	530	1,884	684	300	984	113	400	513	306	207	414	570	59	+58
" 19	"	44	44	16.7		(1,800)	432	2,232	443	1,789	649	250	899	107	334	441	245	196	382	517	54	+57
" 20	6 0	35	29	14.1	66.3	1,750	301	2,051	374	1,677	609	211	820	101	282	383	162	221	442	378	39	+41
" 21	6.6	40	33	14.4		(1,700)	246	1,946	382	1,564	568	216	784	94	288	382	183	199	398	386	40	+26
" 22	11.4	26	15	18.3	62.8	1,660	107	1,767	485	1,282	465	274	739	77	366	443	81	362	724	15	1.5	+11

mols, multiplied by 0.104 represents the expected total hydroxybutyric acid.

On the first 3 days of the period of calorimeter observations, and when the subject received a high protein, high fat diet, the agreement is not good, some 30 or 40 gm. less being excreted than expected from the computation; but for the remaining 5 days the calculation approximately parallels the excretion. Assuming the hydroxybutyric acid analyses to be correct, I can offer no explanation of the discrepancy, unless it be that some was burned without reaction with ketolytic substance. The later close agreement is interpreted as supporting the general method of calculation, and the hypothesis upon which it is based. For the last 5 days, the total calculated hydroxybutyric acid is 193 gm. and the amount excreted, 193 gm. (not including acetone exhaled, which was not determined).

Case No. 740 of Joslin (17).—This case like Subject "Cyril K" discussed above, is one of a very few upon whom sufficient data is recorded to permit satisfactory analysis. Joslin reports among other data the acetone, acetoacetic acid, hydroxybutyric acid, glucose, and nitrogen excretion and the total calories (at rest) for 24 hours. I have increased the latter by 10 per cent to allow for muscular movements and from these values the ketogenic balance has been calculated for 9 days. The details are given in Table VI. The acetone body excretion is fairly satisfactorily accounted for by the excess of ketogenic substance in the mixture burned, though the amounts expected are usually greater than actually excreted. Total calculated for 9 days, 130 gm.; excreted 95 gm. (not including acetone exhaled). The agreement would be better without making the allowance of 10 per cent additional calories.

A very interesting and important point shown by the data of this subject is that his net total carbohydrate tolerance (represented by the net total glucose equivalents, Column 19 in Table VI) remained practically constant throughout the period and that the marked fall in the ketosis was *not* due to an improvement in "tolerance," but solely to the *decrease in total metabolism* (from undernutrition) and in the consequent amount of ketogenic materials in the metabolic mixture. With the fall in the total metabolism, the rate of ketogenic metabolism decrease to a point where

TABLE VI.

Ketogenic Balance of Diabetic, Joslin Case No. 740.

Column groups: (12)–(14) Ketogenic millimols. (15)–(19) Glucose equivalent millimols. (22)–(23) Total β-hydroxybutyric acid (gm.).

(1) Date	(2) O₂ per min. (cc.)	(3) R.Q.	(4) Total calories (110 per cent)	(5) Calories of β-hydroxybutyric acid + extra glucose	(6) Calories of total metabolism	(7) Protein calories, N × 26.5	(8) Calories from fat	(9) Food CH	(10) Urine glucose − food CH = extra glucose	(11) Urine N	(12) Fat calories × 0.363	(13) Protein N × 15	(14) Total	(15) Fat calories × 0.06	(16) Protein N × 20	(17) Total	(18) Extra glucose × 5.56	(19) Net total	(20) Antiketogenic equivalent not glucose × 2	(21) Excess ketogenic	(22) Calculated	(23) Excreted
Apr. 15	212	0.72	1,550	216	1,786	437	1,349	13	26	16.5	490	247	737	81	330	411	145	266	532	205	21.3	24.9
" 16	214.5	0.73	1,600	194	1,794	429	1,375	17	28	16.2	499	243	742	82	324	406	156	250	500	242	25.1	18.9
" 17	212.7	0.72	1,575	115	1,690	255	1,290	0	16	9.6	468	141	612	77	192	269	91	178	356	256	26.6	11.8
" 18	200	0.735	1,490	52	1,542	215	1,327	0	8	8.1	482	121	603	80	162	242	44	198	396	207	21.5	7.9
" 19	190.3	0.755	1,425	57	1,462	180	1,138	0	3	6.8	413	102	515	68	136	204	16	188	376	139	14.4	7.9
" 21	186	0.75	1,380	30	1,420	209	1,067	0	0	7.9	387	118	505	64	158	222	0	222	444	61	6.3	6.5
" 24	182.7	0.736	1,360	25	1,385	241	1,017	5	0	9.1	369	136	505	61	182*	271*	0	271	542*	0	±0	5.4
" 27	187	0.74	1,395	22	1,417	196	1,131	0	0	7.4	411	111	522	68	148	216	0	216	432	90	9.3	4.7
May 1	173.2	0.76	1,300	19	1,319	201	1,028†	0	0	7.6	373	111	487	62	152	214	0	214	428	59	6.1	4.1

* Assuming 5 gm. food CH burned.

† After deducting 90 per cent of alcohol fed.

it no longer exceeded by a large margin the rate of production of ketolytic substance (*i.e.*, the almost stationary total glucose tolerance). The "balance" becoming more favorable, the ketosis declined.

Mosenthal and Lewis' Severe Diabetic E. W. (18).—A woman of 51 years, weight 55 kilos, height 163 cm., with an ulcer of foot and temperature 102°F. Full data are given for urine analyses, including acetone + acetoacetic and hydroxybutyric acid. The metabolism was not determined, and a more or less arbitrary estimate is necessary. In view of the fever, I have taken the high value of 130 per cent of the normal basal for a woman of the same weight and height, and have assumed that it remained constant throughout the period; both are open to considerable error and the results of the calculation can therefore be only rough approximations. The amounts of urine glucose − food carbohydrate = "extra" glucose, derived from protein or glycerol.

1,800 cal. (normal basal) × 130 per cent + cal. value of "extra" glucose + cal. value of total hydroxybutyric acid = cal. of total metabolism.
Cal. of total metabolism − (urine N × 26.5 cal.) = cal. from fat.

The total ketogenic and glucose equivalents are calculated by multiplying by the factors (p. 407) and from the gross total glucose equivalents the value of the "extra" glucose (derived from protein or glycerol and excreted), is subtracted. The total ketogenic millimols − (*net* glucose equivalents × 2) = excess ketogenic millimols. The detailed data for the last 8 days, when the urine collection was complete, are given in Table VII.

The agreement between the calculated total hydroxybutyric acid and the amount excreted is very striking, total calculated for 8 days, 510 gm.; total excreted, 634 gm. Since the estimate of total calories is probably high the difference perhaps indicates either that some of ketolytic substance (glucose) metabolized was "wasted" or that the factors in the calculation are not correct. But with so many unknown or uncertain factors, a closer agreement can scarcely be expected: and the fact that this very severe ketosis can be approximately explained can be interpreted only as confirmatory of the calculations.

Severe Diabetic "Bessie B" of Wilder, Boothby, and Beeler (6).—The remarkably complete observations recently published by these workers, who have also discussed some of their data from the stand-

TABLE VII.

Ketogenic Balance of Severe Diabetic Woman "E. W." (Mosenthal and Lewis).

| Date. | Urine N | Extra glucose. | Calories of glucose + β-acid. | Total metabolism (calculated). | Ketogenic millimols. | | | Glucose equivalents. | | | | | Excess ketogenic. | Total β-acid. | |
(1)	(2)	(3)	(4)	(5)	Fat calories × 0.363. (6)	Protein × 15. (7)	Total. (8)	Fat calories × 0.06. (9)	Protein × 20. (10)	Total millimols. (11)	For glucose × 5 56. (12)	Net total antiketogenic millimols. (13)	(14)	Expected. (15)	Excreted. (16)
														gm.	gm.
Dec. 3	10.8	40	460	2,250	716	162	878	118	216	334	222	224	654	68	66
" 4	9.8	34	368	2,168	692	117	839	114	196	310	189	242	597	62	50
" 5	9.0	26	403	2,203	713	135	848	118	180	298	144	308	540	56	65
" 6	9.6	33	489	2,289	739	144	883	122	192	314	183	262	621	65	78
" 7	10.5	34	648	2,448	788	157	945	130	210	340	189	302	643	67	111
" 8	8.9	32	620	2,420	793	134	927	131	178	309	180	258	669	70	107
" 9	8.7	32	460	2,210	734	132	970	122	174	296	180	232	638	66	73
" 10	9.9	26	498	2,268	739	148	887	122	198	320	145	350	537	56	85

point of the ketogenic balance, would seem to be splendid material for testing our calculations; and possibly they are, though the results show poorer agreement than any previously encountered. I have recalculated the data on this subject, taking the averages for each diet period, the details being given in Table VIII. The calculations are made as in the other diabetic subjects. The total heat produced in 24 hours (Column 10) is taken at 10 per cent over the average of hourly determined rate (Column 9) and to this is added the calorie value of the average total acetone (as hydroxybutyric acid) and of the extra glucose (urine glucose — food carbohydrate). From this sum representing the gross metabolism, the calories from protein ($N \times 26.5$) and from carbohydrate (if any) are subtracted, the remainder being the calories from fat. From these data the ketogenic balance is calculated for each period. The results (Columns 23 and 24) are not satisfactory, showing fair agreement in only four or five out of the eleven periods. Although a rough parallelism is indicated, the error is, with one exception, that the amount of hydroxy-acid excreted is less than expected from the calculation.

Assuming the analytical and dietary data to be correct, there are a number of possible interpretations: (a) That the factors used in calculating ketogenic mols are too high; or (b) that the estimate of total metabolism is too high (unlikely); or (c) that some keto- or hydroxy-acid was burned without the aid of ketolytic glucose. Which of these or other possibilities may explain the discrepancy it is not possible to say. In view of the better agreement in nearly all of the other cases, it seems preferable at present to leave the questions open and to expect that some subjects may occasionally exhibit such exceptional behavior.

Severe Diabetic "Kramer."—This subject was a man of about 25 years, weight 55 kilos, with very severe ketosis, studied by the writer over a long period in 1914 in the Washington University Hospital. Day and night special nurses were provided to secure accurate diet administration and urine collection. Quite complete data are available as to diet and urinary analyses, but the metabolism was not determined, and can only be guessed at. Table IX gives the essential details for 14 days, during the period of highest ketosis. During the period April 13 to 17, on a high protein diet, with very high ketosis the total heat produced is

TABLE VIII.

Ketogenic Balance of Severe Diabetic, "Bessie B.," of Wilder, Boothby, and Beeler.

Period (1)	No. of days (2)	Diet. Protein (3)	Fat (4)	CH (5)	Urine. Glucose (6)	N (7)	Total acetone (8)	Metabolism. Calories per hr. (9)	Calories net. (10)	Per day gross. (11)	Calories from fat. (12)	Ketogenic milli-mols. Fat (13)	Protein (14)	Total (15)	Glucose equivalents. Millimols. Fat (16)	Protein (17)	CH (18)	Deduction for extra glucose (19)	Net total (20)	Antiketogenic equivalent. (21)	Excess ketogenic millimols. (22)	Total hydroxy-butyric acid. Calculated (23)	Excreted (24)
I	18*	49	85	20	36 0.6	10 7	3.53	40.2	1,061	1,166	883	320	110	480	53	214	0	93	151	308	172	18	6.3
II	2†	0	0	0	0.6	5	10.6	(35.0)	924	930	795	289	76	365	48	102	0	3	147	294	71	7	1.1
III	5	47	88	0	16.3	12.4	2.1	35.3	932	1,009	780	283	186	469	47	248	0	87	212	424	45	5	3.8
IV	4	91	99	1	8.4	15.8	3.1	39.2	1,034	1,221	803	292	237	529	48	316	0	238	126	252	277	29	5.6
V	5	163	138	3	3.6	16.7	9.4	39.7	1,048	1,351	908	330	250	580	54	316	0	332	56	112	468	48	16.9
VI	3‡	10	83	15.6	9.1	4.5	0	36.1	953	953	810	294	67	361	49	90	36	0	175	350	11	1	0(?)
VII	4	31	109	14.7	0	13.1	4.50	34.5	911	1,000	721	262	76	338	43	102	82	0	227	454	0	0	5.6
VIII	8	26	151	42.6	8	5.2	4.7	36.4	961	1,067	864	314	78	392	52	104	0	0	156	312	80	8	8.4
IX	28§	0	0	0	7.1	4.6	0.7	39.3	1,037	1,067	945	343	69	412	57	92	0	39	110	220	192	20	1.2
X	4	105	126	3.8	60	2.15	9.2	42	1,114	1,403	992	360	232	592	59	310	0	313	56	112	480	50	16.5
XI	5‖	20	94	12	24 1	5.8	6.1	37.8	1,001	1,097	943	342	87	429	57	116	0	67	106	212	217	22	11.0

* Average of 16 days for total acetone.
† Apr. 18 and 19 only.
‡ May 9, 10, and 11 only.
§ May 27 and 28 only.
‖ June 4 to 8 only.

TABLE IX.

Ketogenic Balance of Severe Diabetic Man, "Kramer."

Date (1)	Diet (2)	Food CH. (3)	Urine N (4)	Urine Glucose (5)	Extra glucose (6)	Calories value glucose + β-acid (7)	Assumed calories (8)	Total metabolism calories (9)	Calories from protein (10)	Calories from fat (11)	Ketogenic millimols Fat calories × 0.363 (12)	Ketogenic millimols Protein N × 13. (13)	Ketogenic millimols Total (12) + (13) (14)	Glucose equivalents Fat calories × 0.09 (15)	Glucose equivalents Protein N × 20. (16)	Glucose equivalents Deduction for extra glucose (17)	Net antiketogenic equivalent net glucose × 2. (18)	Excess ketogenic millimols (19)	Total hydroxybutyric acid Calculated (20)	Total hydroxybutyric acid Excreted (21)
																			gm.	gm.
1914 Apr. 7	Greens.	15	6.9	48	33	257	1,580	1,837	183	1,654	600	103	703	99	138	183	107	596	62	28
" 8	"	15	7.3	50	35	380	1,580	1,960	193	1,767	641	109	750	106	146	194	115	635	66	53
" 13	Meat, fat, greens.	26	19.5	88	62	671	1,896	2,567	516	2,051	745	292	1,037	123	390	336	354	683	71	94
" 14		22	19.7	76	54	637		2,533	522	2,011	730	295	1,025	121	394	300	430	595	62	93
" 15		18	20.2	91	73	835		2,731	535	2,196	797	303	1,100	132	404	406	260	840	87	120
" 16		18	18.0	80	62	711		2,607	477	2,130	773	270	1,043	128	360	345	286	757	79	102
" 17		18	19.7	62	44	655		2,551	522	2,029	736	295	1,031	122	394	245	542	489	51	104
" 28	Eggs, meat, fat, greens.	25	12.4	54	29	250	1,580	1,830	328	1,502	545	186	731	90	248	161	354	377	39	30
" 29		25	16.6	71	46	342		1,922	439	1,483	538	249	787	89	332	256	330	457	47	36
" 30		25	17.5	71	49	339		1,919	465	1,454	527	263	790	87	350	273	328	462	48	33
May 1		24	16.8	75	51	383		1,963	446	1,517	551	252	803	91	336	284	283	520	54	41
" 2	Meat, fat, greens.	25	15.2	69	44	367		1,947	403	1,544	560	228	788	93	304	245	304	484	50	43
" 5		25	15.9	77	52	383	1,580	1,963	421	1,542	560	238	798	93	318	289	214	554	57	40
" 6		25	17.3	71	46	417		1,997	458	1,539	558	260	818	92	346	256	364	454	47	52

estimated at 120 per cent of the normal basal for a man of the same
size; and during the other periods, at the normal basal (1,580
cal.).   The calculations of the ketogenic balance are made in
the same way as described above.   The last two columns of the
table (Columns 20 and 21) show the calculated and excreted total
hydroxybutyric acid.   There is a fairly close parallelism between
the two throughout the periods.   Although in the second period
of high protein diet considerably *more was excreted than expected.*
Possibly this is due to still higher metabolism at this time.   But in
view of the uncertainty as to the total metabolism, the calcula-
tions can be accepted only as rough approximations to illustrate
the probable composition of metabolic mixtures which produce
very marked ketosis in diabetes.   With a tolerance which allowed
the catabolism of only about 150 millimols of *total* glucose (27
gm.) in 24 hours, all fat and ketogenic amino-acids in excess of
such equivalents failed to be oxidized past the stage of β-ketobu-
tyric acid.   Increasing the amount of food protein had two very
undesirable results; added more ketogenic molecules to the mixture
in the form of amino-acids, and probably also stimulated the total
metabolism and thus increased the number of fatty acid molecules.
According to such a point of view the use of high protein diets
in diabetes should theoretically be avoided, a conclusion which is
wholly in accord with the dietetic plan advocated by Newburgh
and Marsh (19) and recently well emphasized by Wilder, Boothby,
and Beeler (6).

Non-Diabetic Subjects on Low Carbohydrate, High Protein Diets.

   Experiments of Higgins, Peabody, and Fitz (20).—The authors
were the subjects and took during 4 days diets very rich in pro-
tein and fat but practically free from carbohydrate.   On the 4th
day, two of the subjects excreted 15.5 and 25.5 gm. of total hy-
droxybutyric acid, which promptly ceased on resuming carbo-
hydrate food.   The urine was analyzed and the resting metabolism
was determined by spirometer and gas analysis at 8 a.m. (before
breakfast), and at 4 p.m. daily.

   The resting metabolism is inadequate for estimating the total
energy exchange of active men, and the latter value must again
be guessed at.   Dr. Higgins in a personal letter has kindly given
me his opinion that his own energy exchange during the experi-

mental period was about 3,300 or 3,400 calories. Since the weights, activity, and diets of the three subjects were approximately the same, we may assume that the total metabolism of each was about the same, 3,400 calories per day. On this assumption the ketogenic balance of H.L.H. and F.W.P. for the 4th diet days may be calculated as follows. Some alcohol taken on the 4th day has been ignored in the calculation.

4th Day of High Protein Diet.

Subject.	Total	Urine N.	Protein	Fat	Ketogenic.			Antiketogenic (glucose × 2).			Calculated excess keto-acid.		Total β-hydroxybutyric acid excreted.
					Fat.	Protein.	Total.	Fat.	Protein.	Total.			
	calories		*calories*	*calories*	*milli-mols*	*milli-mols*	*milli-mols*	*milli-mols*	*milli-mols*	*milli-mols*	*milli-mols*	*gm.*	*gm.*
H.L.H.	3,400	22.8	604	2,800	1,017	342	1,359	336	912	1,248	111	11.5	25.6
F.W.P.	3,400	23.7	628	2,772	1,007	355	1,362	332	948	1,280	82	8.5	15.5

In both cases the amount excreted was considerably greater than the calculated expectation. It is not unlikely that the glucose formed from protein may have been burned soon after the absorption of the amino-acids, and that at other periods of the day, less ketolytic material was therefore available. At any rate the result indicates that high protein diets may actually have *greater ketogenic influence* than would be calculated from their glucose equivalence.

Data are recorded by the authors also for short periods on the morning of the 5th day, before food. The resting oxygen consumption per minute is used to calculate the metabolism and the calculated ketogenic balance may be compared with the total hydroxybutyric acid excreted within these periods.

F.W.P. 7 a.m. to 9 a.m., June 12, before breakfast.

$$\text{Resting metabolism} = (0.267 \text{ liter } O_2 \times 4.68 \text{ cal.} \times 120 \text{ min.}) = 150$$
$$\text{Add 30 per cent for activity} = \underline{45}$$
$$\text{Total metabolism} = 195$$

195 cal. − (1 2 gm. urine N × 26.5) = 163 cal. from **fat.**

	Ketogenic.	Glucose.
	millimols	*millimols*
Protein...............	18 (1.2 × 15)	24 (1.2 × 20)
Fat...................	59 (163 × 0 363)	10 (163 × 0.06)
Total..............	77	34

77 − (34 × 2) = 9 = 0.9 gm. hydroxybutyric acid.
Excreted 1.1 gm.

Applying the same calculation to the other subject, we get the following:

H.L.H. 6.45 to 9.15 a.m.   June 6.   2.5 hrs.

$$\text{Resting metabolism} = (0\ 299\ \text{liter O}_2 \times 4\ 68 \times 150\ \text{min.}) = 210\ \text{cal.}$$
$$\text{Add 30 per cent for activity} = \ 63$$
$$\text{Add cal. value of } \beta\text{-acid excreted } 3.4 \times 4.6 = \ 15$$
$$\text{Total metabolism} = 288$$

288 − (2.5 gm. N × 26.5) 222 cal. from fat.

	Ketogenic.	Glucose.
	millimols	*millimols*
Protein...............	37.5 (2 5 × 15)	50 (2.5 × 20)
Fat...................	80.5 (222 × 0.363)	13 (222 × 0.06)
Total..............	118 0	63

Twice the glucose millimols slightly exceeds the ketogenic millimols, and but little ketosis would be expected, but 3.4 gm. total hydroxybutyric acid were excreted.   However, it is not unlikely that the estimate of the total metabolism is much too low for this subject.

If the total metabolism be taken at 100 per cent over the resting value (because of activity in walking to the laboratory), we obtain close agreement.

(1f) Total metabolism, 435 cal. − 66 = 369 cal. from fat.

	Ketogenic.	Glucose.
	millimols	*millimols*
Protein..............	37.5 (2.5 × 15)	50
Fat..................	134.0 (369 × 0.363)	22
Total..............	171.5	72

171.5 − (2 × 72) = 27.5 = 2.86 gm. expected.

Excreted 3.4 gm.

Which of the above estimates is the more correct it is impossible to say, and the calculation is given chiefly to illustrate the importance of the total metabolism. In short periods variations in the rate of excretion may add to the difficulty of such an analysis. On the other hand, the fact that it is possible to harmonize the figures by the aid of altogether reasonable assumptions as to total metabolism serves to set aside the apparent discrepancy.

Why the third subject of Higgins, Peabody, and Fitz showed no ketosis I am unable to explain, unless perhaps it is due either to a large glycogen reserve which lasted throughout the 4 days of the experiment, or to considerably lower total metabolism. Another possibility cannot, of course, be denied, that this subject burned keto-acid without reaction with ketolytic substance; but I should be inclined to seek some other explanation.

Case of Hubbard and Wright (5).—R. S. H., normal man doing laboratory work. The total metabolism is estimated at 50 per cent over the determined basal, or 1,750 × 150 per cent = 2,624 cal. per day. The largest excretion of acetone, average 11.8 gm. hydroxybutyric acid, was observed on July 21 and 22, the last days of a period on a diet containing 32 gm. of carbohydrate. Assuming this amount of carbohydrate to have been burned evenly throughout the day, the ketogenic balance may be calculated.

2,625 − (12.7 gm. N × 26.5) − (32 × 4.1 cal.) = 2,185 cal. from fat.

	Ketogenic.	Glucose equivalents.
	millimols	*millimols*
Protein..............	190.5 (12.7 × 15)	254 (12.7 × 20)
Fat..................	783.5 (2.158 × 0.363)	130 (2,158 × 0.06)
Carbohydrate.........		178
Total..............	974	562

562 × 2 = 1,124 antiketogenic equivalents.

Ratio keto mols: glucose = 1.7

According to the calculation, there was an excess of antiketo-
genic equivalents; but 11.8 gm. of hydroxy-acid were excreted.
The result may be variously interpreted,—that the food carbo-
hydrate (taken in three meals) was burned more or less quickly
after absorption and thus its ketolytic action in part "wasted,"
that the metabolism was higher than supposed, or that the dis-
crepancy indicates error in the factors of the calculation.   I re-
gard the first or second as the more likely.   The discrepancy is
in the same direction as found with the subjects of Higgins, Pea-
body, and Fitz and is often encountered in analyzing data from
normal subjects on low carbohydrate diets.   The effect of such
nearly "balanced" diets is more ketosis than expected from the
calculations.   Data of this sort appear to be less favorable for
testing the factors of calculation than data from fasting or severe
diabetic subjects.

The calculation of data from diabetics on low carbohydrate
diets very recently reported by Wilder and Winter (7) yields simi-
lar results.   Two cases (A376588 and A375561) with 30 and 14
gm. of carbohydrate in the diets, have a calculated excess of anti-
ketogenic equivalents with consequent expectation of no (or slight)
ketosis.   But the subjects excreted 6.2 and 5.0 gm. of total
acetone.   Both of these subjects had acute infections, naso-
pharyngitis and maxillary sinusitis, and this fact, as noted by
Wilder and Winter, is probably one reason for these discrepancies.

We have seen another instance of the effect of infection, in the
data from the Mosenthal-Lewis case presented above. Apparently
the existence of an infection may in some manner lower the effi-
ciency with which the body utilizes the available antiketogenic
material.   One can imagine that such an effect might result
either from uneven circulatory conditions or from uneven rates
of metabolism during the day and variations in the composition
of the metabolic mixture. It is not at all impossible that
with an infection the metabolism during exercise or at other
times may be much greater (and richer in ketogenic material)
than would be expected from determination of the resting metabo-
lism during brief periods.   Whatever the explanation, the fact
is of practical importance, and I believe is in accord with clinical
experience with diabetes.

A wider margin of safety, in the form of more carbohydrate and of greater carbohydrate tolerance, is needed to avoid ketosis in subjects with acute infections, than in their absence.

Landergren's Subjects (21).—The data contained in an important paper by Landergren on ketosis, would be very valuable for testing the factors of the ketogenic balance, except that the total metabolism must here also be guessed at. A number of different normal subjects (and a few diabetics) were placed on diets which produced in some cases marked ketosis, and the amounts of acetone + acetoacetic acid and β-hydroxybutyric acid were determined. Body weights, in some instances heights, and a description of the subject's activity, allow an approximate estimate of the probable total metabolism. These estimates are admittedly open to considerable errors, which affect especially the amounts of fat burned and consequently the end-results of the calculated ketogenic balance. The details of the "balance" for the normal subjects on the last day of diet periods are shown in Table X. The small amounts of alcohol and of "N-freie Subst." in the diets of some subjects are ignored in the calculations.

The results show some discrepancies, but on the whole the agreement is rather remarkable. The comparison is indicated by the following figures, the first of each pair being the calculated grams of total hydroxybutyric acid and the second the amount excreted: 6.4 to 7.6; 0 to 6.6; 0 to 4.7; 28 to 15.5; 16 to 16.9; 15 to 17.1; 17.0 to 41.8. The differences are here also in the direction of greater excretion than expected. And it is not unlikely that this may be the result of a wasting of ketolytic material by its *periodic* absorption and catabolism after meals, as compared with the more even metabolism of fasting.

It is worthy of note that the remarkably high amount of 41 gm. of hydroxybutyric acid was excreted by a normal man on high protein diet, who indulged in *rigorous muscular activity* throughout the period (28,000 steps per day by pedometer). A very important practical point is here illustrated: that muscular activity under such conditions is done only at the expense of fat metabolism, and that an increase in fat metabolism, if glucose is not available and usable may produce serious ketosis. Bursts of muscular activity would therefore be dangerous in severe diabetes

TABLE X.

Ketogenic Balance of Normal Men on Low Carbohydrate Diets (Subjects of Landergren).

(1) Subject	(2) Body weight (kg.)	(3) Height (cm.)	(4) Day of diet	(5) Diet: Protein	(6) Fat	(7) CH	(8) Assumed total calories	(9) Urine N	(10) Calories from fat burned	(11) Ketogenic: Protein N × 15	(12) Fat calories × 0.363	(13) Total	(14) Glucose: Protein N × 20	(15) Fat calories × 0.06	(16) CH glucose × 3.56	(17) Total	(18) Antiketogenic equivalent (17) × 2	(19) Ketogenic excess millimols	(20) Total hydroxybutyric acid Calculated (gm.)	(21) Exerted (gm.)	(22) Ketogenic ratio ketogene mols: glucose
"Cand. W-D" Normal man, 23 yrs.	73	178	3	122	222	0	*2,800	19.7	2,280	296	828	1,121	394	137		531	1,062	62	6.4	7.6	2.06
"Cand. L-K" Normal man, 23 yrs.	50	166	3	111	222	0	*2,500	21.0	1,940	315	704	1,019	420	116		536	1,072	0	0	6.6	1.9
"Cand. H-N" Normal man, 24 yrs.	74	186	3	120	220	0	3,000	23.0	2,385	348	866	1,214	464	143		607	1,211	0	0	4.7	2.0
"Dr. B.J." Normal physician, 22 yrs.	68		5	2	465	3	2,000	5.7	1,880	85	652	755	114	111	16	241	482	275	28.0	15.5	3.4
"Cand. Sj-gt" Normal man, 27 yrs.	68		5	1	347	2	2,000	9.6	1,750	144	636	780	192	105	11	313	626	154	16.0	16.9	2.5
"Cand. Ekd." Man, 25 yrs. Very active before and during experiment. 25,000 to 28,000 steps per day. Diarrhea and abdominal cramps.	58	171	3	120	220	0	3,000	18.1	2,520	271	915	1,186	362	151		513	1,026	160	17.0	41.0	2.3

or even perhaps in some non-diabetic subjects on low carbohydrate diets.

Such a conclusion agrees with clinical experience in diabetes.

<div align="center">DISCUSSION.</div>

<div align="center">*The Threshold of Ketosis.*</div>

Reviewing the data and results of calculation so far presented, it seems to the writer that the evidence may be regarded strong enough to establish our general theory as the explanation of antiketogenesis. Although a single instance is cited which is possibly altogether exceptional, all of the other subjects developed their ketosis in accordance with a common rule. We are inclined to believe also that the quantitative estimates of the respective ketogenic and antiketogenic influences of fat, protein, and carbohydrate are shown to be approximately, but only approximately, correct. As more reliable data are accumulated considerable revision will doubtless be found necessary; but in the meantime, it may be worth while to see how the conception can be applied to practical problems.

The analyses of the ketogenic balance appear to show less discrepancies when severe ketosis exists, that is to say when there is a large surplus of keto-acid molecules in the body to combine with all available ketolytic molecules. Under such circumstances there appears to be little doubt that each molecule of glucose is equivalent ketolytic substance for 2 molecules of keto-acid.

Where or when such surplus does not exist, it may be imagined that some glucose molecules are oxidized without encountering keto-acid, and thus its ketolytic value is wasted; while in other cells or localities there may be a deficit of glucose with consequent appearance and accumulation of the acetone bodies. Such a situation would result from uneven or unequal distribution of metabolites, just as blockade and hoarding during war time food shortage interfere with the nutrition of some individuals and of some nations, while others have a surplus. This is the state of affairs near the border-line or threshold of ketosis. It may take considerably more than the theoretical amount of total glucose to insure its reaction with every molecule of keto-acid formed in the body, just as it takes a surplus of food to insure the normal

nutrition of all individuals in a community.   The amount of the
excess needed to avoid all ketosis will probably vary, depending
upon rate of absorption and evenness of distribution and metabo-
lism; and therefore, one would perhaps expect the metabolic
mixture of the whole body and its ketogenic ratio to vary at the
threshold of ketosis between the theoretical ratio for the ketolytic
reaction and a considerably higher value.   And this is exactly
what has so far been observed.

The data submitted in earlier papers (2, 3) by the writer indi-
cated a molecular ratio of $\dfrac{\text{ketogenic mols}}{\text{total glucose}}$ = about 1 at the bor-
der-line of ketosis.   The same conclusion was reached by essen-
tially the same methods of calculation by Woodyatt (4) and by
Hubbard and Wright (5).   But more recently Wilder and Winter
(7) cite a number of cases, with very slight ketosis, with ratios
between 1.2 and 2.0.   According to the point of view stated
above, both conclusions can be harmonized.   Some subjects un-
doubtedly show small but definitely abnormal amounts of
acetone when the mixture of metabolites, judged by the respiratory
quotients or by calculation, corresponds to a $\dfrac{\text{ketogenic}}{\text{total glucose}}$ ratio of
about 1:1; while other subjects may have a ratio of almost 2:1
before large amounts of keto- and hydroxybutyric acid appear
in the urine.

In further illustration of this fact, the calculations of some
of the valuable and historically interesting data of Hirschfeld
(1895) might be presented (22).   It will be recalled that Hirsch-
feld first drew the important conclusion that "nicht in Folge
des Hungers und des Zerfalls an Korpereiweiss, sondern nur in
Folge des Fehlens der Kohlenhydrat bei Ernährung erfolgt die
Bildung von Aceton."   From his description of his subjects
(various non-diabetic hospital patients), their body weights, the
diets and their duration, the excretion of urinary total nitrogen,
and acetone plus acetoacetic acid, I have calculated the approxi-
mate ketogenic balance of these patients nearly all of whom ex-
creted only small amounts of acetone and were therefore examples
of border-line ketosis.   The data are rather incomplete and the
tables of calculations need not be recorded, but the keto ratios
obtained are certainly not very erroneous.   The figures show that

on the days when the subjects excreted distinctly abnormal amounts of acetone (more than 0.1 gm.) the amounts of protein, fat, and carbohydrate metabolized were such as corresponded to ratios of ketogenic : glucose mols between 1:1 and 2:1, and in general the higher the ratio the more acetone was excreted. With the ratios of 0.6 to 1.3 and even 1.6, the acetone + acetoacetic excretion was 0.03 to 0.10 gm. acetone, practically normal values, while with ratios from 1.1 to 2.1 up to 0.70 gm. acetone was excreted.

Evidently the range between ratios of 1:1 and 2:1 is variable in that either very small or moderate amounts of total acetone may result.

Practical Significance of the Ketogenic Balance.

The practical interest and significance or the ketogenic balance is that it allows the rational formulation of diets low in carbohydrate and thus designed to spare the carbohydrate tolerance of diabetics from overstrain, and at the same time indicates the minimum amount of total glucose equivalent necessary to avoid the dangers of ketosis. This is the main dietetic problem in diabetes. Apart from the very considerable difficulties of choosing suitable food material, the digestive and other consequences of a restricted diet, the only unusual nutritional limitation imposed by the disease is that the subject must maintain the inflow of all glucose-forming foods *below* the rate at which he can burn it, to avoid further loss of tolerance, and *above* the rate necessary to provide sufficient ketolytic material to avoid ketosis. The width of the margin between these extremes, very great in normal subjects and narrower in diabetics, represents the factor of safety possessed by the individual. The practical question, therefore, is to determine for every diabetic the minimum amount of carbohydrate needed by him for a safe margin over a bare ketogenic balance. So long as this safe margin is maintained, there is no reason to expect ketosis, and the disease would amount to little if anything besides dietary inconvenience. The calculation of a diet to meet this need may be gone about in several ways. Woodyatt (4), Hubbard and Wright (5), and Wilder (8) have already developed valuable formulas for this purpose, based upon

the conception of the ketogenic-antiketogenic balance. The matter is of such importance as perhaps to be worthy of further consideration in the light of the data presented in this paper; we shall first restate the argument as developed in our study of the subject.

If our general idea as to the character of antiketogenesis is correct, it means that the development of ketosis in any subject is wholly determined by the *relative* number of molecules of ketogenic *versus* ketolytic substance in the *mixture being metabolized*. This mixture is made up of a variety of substances formed from the three foodstuffs, protein, carbohydrate, and fat. The composition of the mixture and the resultant of its "ketogenic balance" therefore depend upon those factors which determine how much of each flows into the metabolic stream. Some of these factors are fairly well known and can be predicted, while the effect of others can be determined in individual cases.

Chief among these factors are the diet, the weight or body surface of the subject, his nutritional state, and the degree of muscular activity. The following general statements as to the effect of these factors are based upon well known principles of nutrition, and may perhaps be regarded as almost axiomatic. The diet has only these (immediate) effects:

1. Food protein, *above a certain minimum*, is approximately the amount metabolized (nitrogen equilibrium), provided the total energy value of the diet and the amount of accompanying carbohydrate is not too low. Within certain limits therefore the amount of food protein *determines* the amount of protein metabolized, and its contribution of ketogenic and ketolytic material. The two compensatory quota may, however, not be catabolized simultaneously, and if not, the protective value of the derived glucose may be in part lost. Another important effect of large protein ingestion is its marked stimulation of total metabolism ("specific dynamic action"). Because of its considerable ketogenic influence and its stimulating effect, the lowest possible protein *metabolism* appears to be indicated when ketosis is feared; but on low protein and low carbohydrate diets body protein is apt to be drawn upon, and the total may even be increased. The optimum protein *intake* is doubtless the lowest which will maintain

approximate nitrogen equilibrium;[3] the urinary total nitrogen is the only indication of the amount catabolized, and must be known for the analysis of a ketogenic balance.

2. Food carbohydrate, temporarily stored as glycogen, is oxidized in preference to fat within the carbohydrate tolerance of the subject, beyond which it is excreted as glucose. If taken in larger amounts than required at the time, some may also be converted into fat and stored. Its effect in increasing the rate of metabolism is small.

3. The amount of food fat has only little influence upon the amount of fat burned, which is chiefly determined by the requirement for energy to warm the body and to do its muscular work. If an abundance of available carbohydrate (or protein fragments) meets the greater part of the demand for energy, little fat is burned regardless of whether or not it is eaten; while on the other hand in fasting or on low carbohydrate diets fat is the chief fuel even though none is eaten.

The second of the main factors which influence the metabolic mixture is that set of influences which determine the rate of basal metabolism. In the same category, but quite distinct, is the effect of muscular activity. These factors are decisive in controlling the *amount* of energy exchange and consequently the total amounts of food materials which are burned. On this point the diet has (directly) only a very minor influence. The individual who by hard work expends a total of 3,000 or 4,000 calories a day, obtains that energy from the oxidation of fat, carbohydrate, and protein, even though he eats no food during that time. The same individual at rest in bed would spend only half that energy, and would burn half as much foodstuffs. If we suppose this subject to be fasting, or that the same (small) amount of carbohydrate is available on both days—or that he is a diabetic with a fairly low tolerance—his metabolic mixture and his ketogenic balance would be quite different on the 2 days. At rest the ketolytic fractions might be enough to prevent ketosis, but not enough to dispose of double the amount of keto-acid derived from the additional fat burned to do work.

[3] See the recent paper by Marsh, Newburgh, and Holly (Marsh, P. L., Newburgh, L. H., and Holly, L. E., *Arch. Int. Med.*, 1922, xxix, 97) which indicates that from 0.75 to 1 gm. protein per kilo is usually sufficient.

From the point of view which grows out of the above considerations, the problem of calculating the optimum diet in diabetes appears to focus upon the following question. *How much additional ketolytic substance, in terms of glucose of food carbohydrate must be allowed the subject (of known size, activity, and consequently energy requirements) in order to provide a safe margin over a bare ketogenic balance in his metabolic mixture?* If he has sufficient "tolerance" to burn (utilize) such an amount, and receives it, ketosis will not appear, but if his tolerance is already lower, ketosis is unavoidable until the *amount* of the metabolic mixture (the total metabolism) is decreased to the point where a ketogenic balance is attained.

The equation which must be solved to answer the above question has four main terms, the amounts of protein, of carbohydrate, of fat, and total calories of metabolism. The first two within certain limits may be fixed by diet, while the last is almost wholly determined by the size and activity of the individual. And when the diet is fixed, the amount of fat burned depends upon the total metabolism.

Protein and fat each have both ketogenic and antiketogenic quotas, while carbohydrate has only the latter influence. Accepting for the present the quantitative estimates for these several factors found to yield fair agreement in the analyses of cases presented above, the *net* effect of each may be stated as follows:

Protein, 1 gm. N = (20 glucose equivalents × 2) − 15 ketogenic = 25 millimols excess antiketogenic equivalents.

Fat, 1 gm. = 3.43 ketogenic − (0.57 glucose equivalents × 2) = 2.29 millimols excess ketogenic equivalents.

and 1 cal. from fat = $\frac{1}{9.46} \times 2.29 = 0.242$ millimols excess ketogenic equivalents.

Carbohydrate, 1 gm. glucose = 5.56 × 2 = 11.1 millimols antiketogenic equivalents and 1 cal. from glucose = $\frac{11.1}{3.76} = 2.96$ millimols antiketogenic equivalents.

According to the above values, the excess antiketogenic equivalents of protein corresponding to

1 gm. of urinary nitrogen would "neutralize" $\frac{25}{0.242} = 103$ cal. from fat.

And 1 cal. from glucose would "neutralize" $\frac{2.96}{0.242} = 12.2$ cal. from fat.

And,

$$\begin{bmatrix} \text{Total calories} \\ \text{of metabolism} \end{bmatrix} - \begin{bmatrix} \text{Calories from} \\ \text{protein} \end{bmatrix} - \begin{bmatrix} \text{Calories of fat ``neutralized'' by} \\ \text{excess antiketogenic from protein} \end{bmatrix} =$$

$$\begin{bmatrix} \text{Calories of metabolism not ``neutralized'' by} \\ \text{antiketogenic of protein and fat, and which} \\ \text{must be supplied from fat and glucose in} \\ \text{ratio of not more than 12.2 fat cal. to 1 glu-} \\ \text{cose cal.; or glucose calories must be at least} \\ \dfrac{1}{12.2+1} = 0.076 \text{ of the total unneutralized} \\ \text{calories.} \end{bmatrix}$$

On substituting values for the above expressions, the equation becomes

$$\frac{[\text{Total cal.} - (N \times 26.5) - (N \times 103)] \times 0.076}{3.76 \text{ cal.}} = \text{gm. glucose}$$

necessary to provide theoretical ketogenic balance.

Simplified and the values rounded off,

$$\frac{\text{Total cal.} - (N \times 130)}{50} = \text{Minimum extra glucose (gm.).}$$

According to this expression whenever the urine nitrogen multiplied by 130 equals or exceeds the total calories of metabolism, no carbohydrate is needed to provide a theoretical balance. Such conditions may be thus illustrated.

With   5 gm. urine N, the total metabolism might be   650 calories.
"   10 "   "   "   "   "   "   "   " 1,300   "
"   15 "   "   "   "   "   "   "   " 1,950   "
"   20 "   "   "   "   "   "   "   " 2,600   "
"   30 "   "   "   "   "   "   "   " 3,900   "

These figures perhaps indicate the way in which the Eskimo may avoid ketosis without carbohydrate food.

But this calculation assumes an even metabolism and the maximum utilization of all ketolytic substance, which is doubtless rare.   The results cited above from subjects on high protein diets, or near the border-line of ketosis indicate that a variable but considerable excess of food carbohydrate is usually necessary.   The amount of this excess may vary with the subject, with the frequency of its ingestion, with the "peaks" of muscular activity and metabolism, or with other unknown factors; but it appears that double the quantity is usually sufficient.

A slightly different equation developed from a previous analysis (10) is perhaps preferable to the one stated above because somewhat simpler, easier to remember and to calculate.   Since both

equations give the *theoretical minimum* of food carbohydrate, and
a variable margin over this minimum is almost always necessary,
the simpler formula is doubtless sufficiently accurate.   This
formula is

$$\frac{\text{Total calories of metabolism} - (\text{Urine N} \times 100)}{50} = \text{Minimum food carbohydrate (gm.).}$$

It is suggested that this equation be used as follows.   Estimate
from the size, weight, and activity of the subject his approxi-
mate metabolism for 24 hours.   If necessary determine the urine
nitrogen on the amount of food protein decided upon; or tenta-
tively assume nitrogen equilibrium on 0.75 to 1.0 gm. food protein
per kilo, and confirm it later after diet is established.   Include in
the diet the calculated minimum amount of starch, or preferably
double this quantity (or more if the diabetes is not severe).
Supply the rest of the calories needed in the form of fat.
On theoretical grounds the food should be evenly distributed
throughout the day in frequent small meals.

If on such an "optimum" diet thus determined the subject
shows glycosuria and ketosis, it obviously means that the carbo-
hydrate tolerance is lower than *necessary* for the *amount* of the
materials metabolized, and the *only* recourse is to *decrease* the
total metabolism.   And this can be accomplished only by mus-
cular rest or by undernutrition or both.

The undoubted benefit of fasting, advocated by Allen, probably
has its explanation in this lowering of the total metabolism, as
pointed out by Allen and Du Bois (23).   The more recent use of
low protein and (moderate) fat diets of Newburgh and Marsh (19)
also appears to be theoretically sound; and the still more recent
proposal by Wilder (8) of diets about 25 per cent below the require-
ment, and the points of view of the latter and of Woodyatt (4) are
in harmony with our conclusions.   The emphasis which both have
placed upon the materials *metabolized*, rather than on the diets
fed, is wholly in accord with the conception underlying our work
and is the basis on which further progress in the dietary treat-
ment of diabetes may be expected.

BIBLIOGRAPHY.

1. Shaffer, P. A., *J. Biol. Chem.*, 1921, xlvii, 433.
2. Shaffer, P. A , *J. Biol. Chem.*, 1921, xlvii, 449.
3. Shaffer, P. A., *J. Biol. Chem.*, 1921, xlix, 143.
4. Woodyatt, R. T., *Arch. Int. Med.*, 1921, xxviii. 125.
5. Hubbard, R. S., and Wright, F. R., *J. Biol. Chem.*, 1922, l, 361.
6. Wilder, R. M., Boothby, W. M., and Beeler, C., *J. Biol. Chem.*, 1922, li, 311.
7. Wilder, R. M., and Winter, M. D., *J. Biol. Chem.*, 1922, lii, 393.
8. Wilder, R. M., *J. Am. Med. Assn.*, 1922, lxxviii, 1878.
9. Benedict, F. G., A study of prolonged fasting, *Carnegie Inst. Washington, Pub. 203*, 1915.
10. Shaffer, P. A., *J. Biol. Chem.*, 1922, l, p. xxvi.
11. Gephart, F. C., Aub, J. C., Du Bois, E. F., and Lusk, G., *Arch. Int. Med.*, 1917, xix, 908.
12. Means, J. H., *J. Med. Research*, 1915, xxxii, 121.
13. Folin, O., and Denis, W., *J. Biol. Chem.*, 1915, xxi, 183.
14. Grafe, E., *Z. physiol. Chem.*, 1910, lxv, 21.
15. Bönniger, M., and Mohr, L., *Z. exp. Path. u. Therap.*, 1906, iii, 675.
16. Brugsch, T., *Z. exp. Path. u. Therap.*, 1905, i, 419.
17. Joslin, E. P., *Am. J. Med. Sc.*, 1915, cl, 490.
18. Mosenthal, H. O., and Lewis, D. S., *Bull. Johns Hopkins Hosp.*, 1917, xxviii, 187.
19. Newburgh, L. H., and Marsh, P. L., *Arch. Int. Med.*, 1920, xxvi, 647.
20. Higgins, H. L., Peabody, F. W., and Fitz, R., *J. Med. Research*, 1916, xxxiv, 263.
21. Landergren, E., *Nord. Med. Arch.*, 1910, xliii, Afd. II, Inre Medicin, No. 10.
22. Hirschfeld, F., *Z. klin. Med.*, 1895, xxviii, 176.
23. Allen, F. M., and Du Bois, E. F., *Arch. Int. Med.*, 1916, xvii, 1010.

THE ESTIMATION OF FORMIC ACID IN THE URINE.

By ETHEL M. BENEDICT and G. A. HARROP.

(*From the Department of Medicine of the College of Physicians and Surgeons of Columbia University, and the Presbyterian Hospital, New York, and the Chemical Division, Medical Clinic, the Johns Hopkins University and Hospital, Baltimore.*)

During the course of some clinical and experimental studies of acute methyl alcohol poisoning (1) the necessity arose for making a considerable number of quantitative estimations of the excretion of formic acid in the urine. The method at first employed was that of Dakin, Janney, and Wakeman (2), which is satisfactory but very time-consuming. It was found desirable to devise a simpler and quicker procedure, which would obviate if possible the necessity for extraction with ether and shorten the time required for the other manipulations. The following method has proved to be satisfactory in our hands and yields better results than are obtained by the ether extraction method.

Methods.

100 cc. of urine are accurately measured into a liter volumetric flask containing 500 to 600 cc. of water. 100 cc. of 20 per cent $CuSO_4$ are added and the whole is well mixed. A 10 per cent suspension of $Ca(OH)_2$ is then added until alkalinity, as evidenced by the characteristic color change from green to blue, occurs. It is well to avoid an excess of calcium hydroxide. The contents are then made up to volume and mixed. After standing 15 to 30 minutes they are filtered and a 600 cc. aliquot is placed in a 800 cc. Kjeldahl flask, with 2 to 3 drops of phenolphthalein as an indicator, and made distinctly acid with 85 per cent phosphoric acid.[1] Sufficient excess should be added to free all of the formic acid present (1 to 2 cc. are ample for this purpose). Glass beads are

[1] Tartaric acid may be used instead of phosphoric, but we have not found that it has any particular advantage. Strong mineral acids cannot be used.

443

added to prevent bumping. This flask is connected to a supply of steam and to a water condenser by means of a Kjeldahl trap. Both the neck of the flask and the outside of the trap are well wrapped with asbestos cord to prevent loss of heat.[2] The distillate is caught in a casserole provided with 15 to 20 cc. of 0.1 N NaOH, and a few drops of phenolphthalein are added to make sure that it is alkaline. This alkalinity must be maintained throughout the distillation by the addition of further 0.1 N NaOH as may be necessary, but a great excess must be avoided. It is neither necessary nor advisable to cause the delivery tube from the condenser to drop under the liquid in the casserole. The distillation must be so managed that at first a slow stream of steam is conducted in, while the contents of the distilling flask are rapidly driven over by brisk heating and reduced in volume to 50 to 75 cc. At this amount they must remain until 2 liters are collected.[3]

The distillate is then evaporated to dryness over night on the water bath, and the residue taken up in exactly 100 cc. of distilled water and filtered. A 90 cc. aliquot is placed in a 250 cc. Erlenmeyer flask and made just acid with 0.1 N HCl. 10 cc. of the special HgCl₂ mixture[4] are then added and the flask is fitted with an air condenser and heated in a boiling water bath for 1 hour. After cooling the mercurous chloride is filtered into a weighed Gooch crucible, washed with 100 cc. of 5 per cent cold HCl, then water, alcohol, and ether, and dried 1 hour at 105° and weighed. The blank of the reagents in our experiments has varied from

[2] Such a device is essential, as otherwise phosphoric acid is mechanically carried over during the rapid distillation and causes much trouble. It is convenient to do the steam distillations on a Kjeldahl rack.

[3] This reduction in volume is necessary because the amount of formic acid carried over by steam distillation varies enormously with the volume of the distilling fluid. On the other hand, if the volume is allowed to become too low, especially if it boils dry, large errors result from the breaking down of other organic substances present into formic acid. 2 liters of distillate are sufficient to collect if these precautions are observed. It should be collected in about 2 hours.

[4] This solution contains 200 gm. of HgCl₂, 80 gm. of NaCl, and 300 gm. of Na acetate to 1 liter of water (3). 10 cc. of this solution are sufficient for amounts of formic acid up to 0.13 gm., that is a concentration sixteen times the upper limit in normal urine. For urines containing larger amounts, larger amounts of this mercuric chloride mixture must be used.

0.0014 to 0.0014 gm. according to the reagent used. The amount of formic acid per liter in the original urine is then 1.01 $\times \dfrac{10}{6} \times \dfrac{10}{9} \times 0.0975 \times$ (weight of precipitate $-$ weight of blank of reagents).[5]

DISCUSSION.

As has been frequently pointed out, the isolation of volatile fatty acids by steam distillation direct from acidified urines often entails serious errors, because the prolonged action of mineral acid upon carbohydrates and other substances present, results in fatty acid, especially formic acid formation.[6] The precipitation with copper hydroxide, a device introduced by Van Slyke in his well known acetone body methods (4), disposes of this difficulty by removing the sugar and other interfering substances. The steam distillation should be commenced at once on the filtrate and its reduction in volume effected in the process. It is a waste of time first to try to concentrate the alkaline aliquot and it may cause considerable error because of the destruction of organic acids which takes place when they are heated in alkaline solution. Further, it has been found that when formaldehyde is present (as is the case in urines obtained during acute methyl alcohol poisoning), about 10 per cent will be oxidized to formic acid in the process of evaporating in alkaline solution.

The addition of NaCl and of Na(CH₂COO) to the HgCl₂ solution hastens very greatly the precipitation of HgCl and also prevents the precipitation of impurities. As seen in the curve (Fig. 1). 99 per cent is precipitated after 1 hour heating, at the end of which the reaction is stopped and 1 per cent added to the final figure of the result. We have spent a great deal of time in efforts to develop a method for titrating the mercury, but we believe the gravimetric estimation is more reliable and satisfactory.

[5] 1 gm. of HgCl equals 0 0975 gm. of formic acid.

[6] It may be pointed out that this error obtains in the Ryffel method for estimating lactic acid, (i.e.. steam distillation from urine to which equal parts of concentrated H_2SO_4 have been added), when determinations are made upon urines containing carbohydrates. Treatment with $Cu(OH)_2$ will obviate this error in such lactic acid determinations.

Determinations of the formic acid content of urines should be commenced as soon as possible after collection. We have found it quite impossible even when kept on ice or when preservatives are used, to get concordant results when specimens stand over 24 hours.

Tables I and II indicate how completely known formic acid can be recovered from pure aqueous solutions and from urine by this method.

Fig. 1. Curve showing the rate of formation of mercurous chloride precipitate.

The Occurrence of Formic Acid in Human Urine.

A number of analyses of the formic acid content of normal urine from individuals on mixed diet are given in the literature. The amount is small and is given by Dakin, Janney, and Wakeman, as varying from 29.9 to 118 6 mg. per 24 hours. Our results are in agreement with these figures.

A number of analyses on urines from patients which show a high organic acid content are given in Table III. It is known that an increase in the consumption of carbohydrates causes an increased formic acid excretion, a condition which would also presumably result from administration of glucose intravenously

TABLE I

Recovery of Formic Acid from Aqueous Solutions.

Date.	Amount of formic acid added.	Amount of formic acid found.	Error	
	gm.	*gm.*	*gm.*	*per cent*
July 7, 1920	0.2404	0.2364	0.0040	1.6
	0.2404	0.2401	0.0003	0
	0.2404	0.2442	0.0038	1.5
June 10, 1920	0.1202	0.1198	0.0004	0
	0.1202	0.1215	0.0011	0.9
	0.1202	0.1216	0.0012	1.0
" 14, 1920	0.6010	0.5821	0.0189	3.0
	0.6010	0.6035	0.0019	0.3
	0.6010	0.5835	0.0175	2.9
" 15, 1920	0.0060	0.0064	0.0004	
	0.0060	0.0064	0.0004	
	0.0060	0.0070	0.0010	
" 21, 1920	0.6010	0.5904	0.0106	1.8
	0.6010	0.5948	0.0062	1.3
May 9, 1922	0.1208	0.1218	0.0010	0.8
	0.1208	0.1203	0.0005	0.4

TABLE II.

Recovery of Formic Acid Added to Urine.

Date.	Subject.	Amount in urine.	Amount added.	Amount recovered.	Error.	
		gm per 100 cc	*gm.*	*gm.*	*gm.*	*per cent*
June 22, 1920	A	0.0053				
		0.0060				
	Nephritic with albuminuria.		0.0240	0.0236	0.0004	1.9
			0.0240	0.0229	0.0011	4.8
			0.6010	0.6043	0.0033	0.5
			0.6010	0.6127	0.0117	1.9
			0.6010	0.5896	0.0114	1.9
Oct. 29, 1920	B	0.0073				
	Diabetic, 1 0 per cent sugar. Acetone and Diacetic + + + +		0 0240	0.0248	0.0008	3.0
			0 6010	0.5992	0.0018	0.3
May 5, 1922	C	0.0038				
		0.0040				
	Diabetic, 3 0 per cent sugar. Acetone and Diacetic + + + +		0.1208	0.1167	0.0041	3.4
			0.1208	0.1170	0.0038	3.1
Apr. 19, 1922	D	0.0019				
		0.0026				
	Normal individual.		0.1208	0 1167	0 0041	3.4

TABLE III.

Excretion of Formic Acid in the Acidosis of Starvation and of Diabetes.

Name	Date	0.1 N organic acids*		0.1 N formic acid		0.1 N acetone bodies.		0.1 N acid not accounted for.*		Blood CO₂.	Condition.
	1922	Per liter	Total.	Per liter	Total.	Per liter	Total.	Per liter	Total.	vol. per cent	
		cc.	cc.	cc.	cc.	cc.	cc.	cc.	cc.		
E	Apr. 19	488	968	5	9.3	225	418	145	270		Epileptic, starvation.
F	June 2	1,576	2,459	9.6	15	932	1,452	438.1	687	23.6	"
F	" 3	1,706	1,963	4.5	5.2	919	1,057	537.5	620.8		"
S	" 5	1,290	2,472	10.5	21.5	694	1,430	335.5	690.5		"
H	May 29	874.1	2,884	17.6	58	468	1,547	347.6	1,115	27.9	Diabetes, acidosis.
H	June 15	534	2,295	14	60	225	968	251	1,081	33.4	"
L	" 20	1,552	3,107	16	32.5	935	1,889	446	872	21.0	"

* Creatinine and creatine have been corrected for in the "acids not accounted for" but not in the total organic acids.

or by other routes. The data, however, indicate that an increased excretion of formic acid does not commonly occur in the acidosis of diabetes. An opportunity to study the excretion in the acidosis of starvation was afforded by the analysis of urines from epileptic patients under treatment. The data indicate that formic acid excretion plays no part in the production of the high organic acid excretion usually found in these fasting patients.

TABLE IV.

Excretion of Formic Acid in Experimental Methyl Alcohol Poisoning.

Dog K. Weight 7.2 kilos. Male.

Date.	Urine.	0.1 N organic acid.	0.1 N formic acid.	Remarks.
1920	cc.	cc.	cc.	
May 3	240	271	23	
" 4	600		24	
" 5	290	250	15	Given 8 gm. CH$_3$OH per kilo by stomach tube.
" 6	650	468	170	
" 7	275	1,234	532	
" 8	195	1,249	514	
" 9	175	1,929	702	
" 10	200	1,625	516	
" 11	395	1,292	671	
" 12	405	871	266	
" 13	101	807	245	
" 14	380	541	188	
" 15	300	511	51	
" 16	410		19	
" 17	310	299		

The one pathological condition in which formic acid excretion has been found to play a predominant rôle is in the acidosis of methyl alcohol poisoning. In Table IV is given the formic acid excretion in one experiment following ingestion of methyl alcohol by mouth.

SUMMARY.

An improved and simplified method for the estimation of formic acid in urine is described.

No increase in the excretion of formic acid has been found during the acidosis due to diabetes, and in that due to starvation. The

formic acid excretion and its relation to the total organic acid
excretion are presented from an experiment in the production of
acute methyl alcohol poisoning.

BIBLIOGRAPHY.

1. Harrop, G. A., and Benedict, E. M., *J. Am. Med. Assn.*, 1920, lxxiv, 25.
   Palmer, W. W., and Harrop, G. A., *Proc. Am. Soc. for Clin. Investiga-
   tion*, *J. Am. Med. Assn.*, 1920, lxxiv, 1669.
2. Dakin, H. D., Janney, N. W., and Wakeman, A. J., *J. Biol. Chem.*, 1913,
   xiv, 341.
3. Fincke, H., *Biochem. Z.*, 1913, li, 253.
4. Van Slyke, D. D., *J. Biol. Chem.*, 1917, xxx, 347.

THE COMPARATIVE TOXICITY OF AMMONIUM SALTS.

By FRANK P. UNDERHILL and ROBERT KAPSINOW.

(From the Department of Pharmacology and Toxicology, Yale University, New Haven.)

In view of the important rôle played by ammonia and ammonium salts in the metabolism of living tissue, this investigation was undertaken to determine the comparative toxicity of the more common ammonium salts, both organic and inorganic.

Rachford and Crane[1] attempted to study the relative toxicity of ammonium compounds and their action in acid intoxication in mice. They found, employing 5 per cent solutions, that the toxicity of the different salts apparently had little or no relation to the ammonia content of the salt (see Table I).

It appears from Tables I and II that the toxicity of ammonium salts depends rather upon the amount of salt present than upon the NH_3 content. This lack of uniformity has been the incentive to repeat this work.

Method.

The animals used were adult white rats of average weights. All the salts used were made to approximate 5 per cent solution. The nitrogen in the form of NH_3 was determined in three 5 per cent solutions by an abbreviated Folin-Farmer microchemical method[2] for total nitrogen. 5 cc. of the ammonia solution were matched against 5 cc. of standard ammonium sulfate containing 1 mg. of nitrogen after being nesslerized as nearly as possible at the same time. In this way it was possible to determine accurately the amount of nitrogen in the form of NH_3 present in 1 cc. and to rule out the weight of the water of crystallization.

[1] Rachford, B. K., and Crane, W. H., *Med. News.* 1902, lxxxi, 778.
[2] Folin, O., and Farmer, C. J., *J. Biol. Chem.*, 1912, xi, 493.

Six animals were used for each series, the amount of solution injected ranged from 0.5 to 3.0 cc. per 100 gm. of body weight. The minimum lethal dose taken was that amount which caused death within 3 hours.

TABLE I.

(Rachford and Crane.)

Arranged to Emphasize Fatal Dosage.

Salt.	Toxicity of 5 per cent solution.	Amount in fatal dose.	Amount of NH₃ in fatal dose.
	cc.	gm.	gm.
Ammonium oxalate.........	0 04	0 002	0.00058
" carbonate..........	0.12	0.006	0.00225
" chromate.........	0.12	0 006	0.00142
" chloride..	0.19	0.009	0.00306
" nitrate	0.19	0.009	0.00202
" citrate.................	0.19	0.009	0.00225
" sulfate.................	0 22	0.011	0.003
" tartrate..............	0 28	0.013	0.0025
" hydrogen phosphate.	0 28	0.013	0.0035
" acetate	0 49	0.026	0.00008
" sarcolactate..........	0.62	0.032	0.00504
" lactate...............	0.74	0.039	0.00655

TABLE II.

(Rachford and Crane.)

Arranged to Show Relationship of NH₃ Content to Fatal Dose.

Salt.	Amount of NH₃ in fatal dose.	Fatal dose.
	gm.	cc.
Ammonium lactate	0.00655	0.74
" acetate	0 00008	0.49
" sarcolactate......	0 00504	0.62
" hydrogen phosphate	0 0035	0.28
" chloride	0 00306	0.19
" sulfate	0 003	0.22
" tartrate	0.0025	0.28
" citrate	0.00225	0.19
" carbonate .	0 00225	0.12
" nitrate .	0 00202	0.19
" chromate.	0 00142	0.12
" oxalate	0 00058	0.04

TABLE IIIa.

The Comparative Toxicity of Ammonium Salts (Inorganic).

Rat series.	Weight.	Substance injected.	Dose per 100 gm. in minimum lethal dose.	Minimum lethal dose.	N in total minimum lethal dose.	N per dosage.	N per 1 cc.	Total active time of minimum lethal dose.	Remarks.
	gm.		cc.	cc.	mg.	mg.	mg.	min.	
A	340	$(NH_4)_2CrO_4$	2.0	6.80	0.07235	0.02128	0.01064	155	
B	270	NH_4Cl	2.5	6.74	0.07162	0.026575	0.01063	17	
C	207	$(NH_4)_2HPO_4$	3.0	6.21	0.06417	0.02467	0.00989	70	
D	225	No. 2 $NH_4(H)CO_3$	2.5	5.60	0.05488	0.0245	0.0098	68	Duplicate.
E	272	NH_4NO_3	2.5	6.75	0.06513	0.024125	0.00965	32	
F	400	$(NH_4)_2SO_4$	2.5	10.00	0.0943	0.023575	0.00943	40	
G	315	No. 1 NH_4HCO_3	2.5	7.87	0.07264	0.023075	0.00923	42	
H	250	NH_4Br	2.5	6.20	0.05487	0.022125	0.00885	91	
I	210	$(NH_4)_2CO_3$ + carbamide.	3.0	6.30	0.055377	0.02637	0.00879	24	
J	160	$(NH_4)_3PO_4$ neutral.	3.0	4.80	0.0384	0.024	0.00800	49	
K	252	$(H_2)NH_4PO_4$	3.0	7.56	0.05708	0.02265	0.00755	83	
L	200	NH_4I	3.0	6.00			0.00672		No deaths. Maximum dose.
M	245	NH_4CrSO_4	3.0	7.40			0.002195		" "

Observations.

It will be seen from Tables IIIa, IIIb, IVa, and IVb that in general the toxicity of the ammonium salts is directly proportional to the amount of NH_3 present, the fatal acting time remaining inversely to the amount of NH_3 present to the number of cubic centimeters injected.

Table IIIa.—The minimum lethal dose and the milligrams of nitrogen in the total minimum lethal dose depend upon the weight of the animal. It is readily seen that the milligrams of nitrogen per 1 cc. tend to run parallel to the dose per 100 gm. in the minimum lethal dose.

Table IIIb.—This table is arranged to show the relation of the milligrams of nitrogen in the total minimum lethal dose to the fatal acting time. Here one can see that the fatal acting time is decreased the greater the amount of NH_3 present.

TABLE IIIb.
The Relation of Toxicity to NH_3 Content.

Rat series	Substance.	N per dosage.	Amount	Time.	Remarks.
		mg.	*cc.*	*min.*	
1	NH_4I		3.0		No deaths recorded with maximum dose.
2	NH_4CrSO_4		3.0		No deaths recorded with maximum dose.
3	$(NH_4)_2CrO_4$	0.02128	2.0	155	
4	NH_4Br	0.022125	2.5	91	
5	$(H_2)NH_4PO_4$	0.02265	3.0	83	
6	No. 1 NH_4HCO_3	0.023075	2.5	42	
7	$(NH_4)_2SO_4$	0.023575	2.5	60	
8	$(NH_4)_3PO_4$ neutral.	0.024	3.0	49	
9	NH_4NO_3	0.024125	2.5	32	
10	No. 2 $NH_4(H)CO_3$	0.0245	2.5	68	Duplicate.
11	$(NH_4)_2CO_3$ + carbamide.	0.02637	3.0	24	
12	NH_4Cl	0.026575	2.5	17	
13	$H(NH_4)_2PO_4$	0.029670	3.0	70	

Exceptions to the above rule are $(NH_4)H_2PO_4$, $(NH_4)_2HPO_4$, and $(NH_4)_3PO_4$; $(NH_4)HCO_3$ and $(NH_4)_2CO_3$ plus carbamide. Two series of $(NH_4)HCO_3$ were carried through because of its rapid deterioration. It was found that although there was a

TABLE IVa.

The Comparative Toxicity of Ammonium Salts (Organic).

Rat series.	Weight.	Substance injected.	Dose per 100 gm. in minimum lethal dose.	Minimum lethal dose.	N in total minimum lethal dose.	N per dosage.	N per 1 cc.	Total acting time of minimum lethal dose.	Remarks.
	gm.		cc.	cc.	ml.	ml.	ml.	min.	
I	220	Ammonium formate.	2.0	4.4	0.0472	0.02146	0.01073	117	
II	400	" acetate.	1.5	6.0	0.01776	0.01194	0.00796	96	
III	280	" citrate.	3.0	8.4			0.00781		No deaths. This was a maximum dose of 5 per cent solution.
IV	260	"	2.5	6.3	0.1029	0.4075	0.01634	38	10 per cent solution.
V	300	" sulfate.	2.5	4.5	0.0257	0.0143	0.00572	63	
VI	320	" lactate.	3.0	9.6			0.004564		No deaths. This was a maximum dose of 5 per cent solution.
VII	200	"	3.0	6.27	0.08652	0.0414	0.0138	86	10 per cent solution.
VIII	360	" benzoate.	2.5	9.0	0.02728	0.00758	0.003032	134	
IX	385	" tartrate.	3.0	11.6	0.02337	0.00762	0.00254	58	
X	480	" valerianate.	3.0	14.4			0.00251		No deaths. Maximum dose of 5 per cent solution.

delay in the fatal acting time or in other words a decrease in
toxicity, nevertheless for the carbonate group, the same rule held
true as with all the other salts. The phosphates tend to run in
the same way although not so perfectly. The importance of these
two types of substances in neutralizing acid bodies of intoxication
demonstrates the relative resistance that the organism has to
them.

Table IVa.—Here the ratio of NH_3 content to dosage and its
relation to the fatal acting time is more clearly demonstrated.
The exceptions in this group are ammonium citrate and ammonium
lactate, but when taken in 10 per cent solutions it is seen that
ammonium citrate containing more NH_3 requires less dosage and
is more actively fatal than ammonium lactate. The resistance to
ammonium lactate may be explained perhaps by the ability of the
body to oxidize lactic acid.

TABLE IVb.
The Relation of Toxicity to NH_3 Content.

Rat series	Substance.		N per dosage.	Amount.	Time.	Remarks.
			mg.	*cc.*	*min.*	
1	Ammonium valerianate.		0.00251	3.0		No deaths. Maximum dose of 5 per cent solution.
2	"	tartrate.	0.00254	3.0	58	
3	"	benzoate.	0.003032	2.5	134	
4	"	lactate.	0.004564	3.0		No deaths. Maximum dose of 5 per cent solution.
5	"	salicylate.	0.00572	2.5	63	
6	"	citrate.	0.00781	3.0		No deaths. Maximum dose of 5 per cent solution.
7	"	acetate.	0.00796	1.5	96	
8	"	formate.	0.01073	2.0	117	
9	"	lactate.	0.0138	3.0	86	10 per cent solution.
10	"	citrate.	0.01634	2.5	38	10 per cent solution.

In comparing the action of NH_3 in the organic and inorganic compounds with regard to a possible diminution of toxicity in the organic compounds to that of the inorganic compounds, it would seem that the organic compounds are less toxic, probably due to the greater ease with which they can be converted to urea.

SUMMARY.

1. The toxicity of ammonium salts is directly proportional to the amount of NH_3 present.

2. The greater the ratio of NH_3 to the salt, the smaller is the minimum lethal dose.

3. The fatal acting time is inversely proportional to the amount of NH_3 present.

THE INFLUENCE OF WATER INTRODUCTION UPON BLOOD CONCENTRATION INDUCED BY WATER DEPRIVATION.

By FRANK P. UNDERHILL and ROBERT KAPSINOW.

(From the Department of Pharmacology and Toxicology, Yale University, New Haven.)

That changes in blood concentration are of considerable significance in various normal and pathological states is rapidly gaining recognition. Disturbances in water balance introduce factors, hitherto unrecognized, which profoundly effect the circulation and hence indirectly exert a striking influence upon the processes of internal respiration leading eventually to more or less marked embarrassment of the vital activities of the cell, and if continued for a sufficient period death inevitably follows.[1]

In connection with another problem it became essential to know the influence of water deprivation for relatively short periods upon alterations of blood concentration and in particular the immediate effect of the introduction of large volumes of fluid upon the changed blood concentration. This problem is the subject of the present communication.

Methods.

Female dogs, full grown and in good nutritive condition, were kept in metabolism cages without food and water for periods

[1] For literature on changes in blood concentration consult: Sellards, A. W., The principles of acidosis, and clinical methods for its study, Cambridge, 1917; Underhill, F. P., The lethal war gases, physiology and experimental treatment, New Haven, 1920; *The Harvey Lectures*, 1918–19, xiv. 234; Underhill, F. P., and Ringer, M., *J. Am. Med. Assn.*, 1920, lxxv, 1531; Marriott, W. McK., *Am. J. Dis. Child.*, 1920, xx, 461; Underhill, F. P., and Greenhouse, B., *J. Urol.*, 1921, vi, 83; Rowntree, L. G., *Physiol. Rev.*, 1922, ii, 116; Underhill, F. P., and Ringer, M., *J. Pharmacol. and Exp. Therap.*, 1922, xix, 163, 179; Underhill, F. P., and Errico, L., *J. Pharmacol. and Exp. Therap.*, 1922, xix, 135.

varying from 5 to 8 days. During this interval blood concentration changes were followed three times daily by estimation of hemoglobin content of blood (method of Cohen and Smith) obtained by puncture of an ear vein. Previous investigations from this laboratory have demonstrated that such estimations furnish a reliable guide for this purpose. Urine volume was determined daily by catheterization. When blood concentration had seemingly reached a maximum water was offered to the animals and they were allowed to drink until satisfied. Hemoglobin estimations were then made at short intervals. During the course of the water deprivation the animals were quiet and apathetic and lost weight very rapidly. After water and food were resumed the dogs quickly regained their weight and normal condition.

The Influence of Short Intervals of Water Deprivation upon Changes in Blood Concentration.

During the progress of this investigation Keith[2] reported marked changes in blood volume in dogs deprived of water for periods varying from 2 to 4 weeks. Hemoglobin was usually but not invariably increased. Blood pressure was also at times markedly lowered. Administration of food and water resulted in a rapid increase in the amount of circulating plasma, a fall in hemoglobin and a decrease in the viscosity of the whole blood, plasma, and serum. The demonstration of a decrease in the volume of the circulating blood offers an explanation for similar clinical conditions such as chronic shock-like states and atrophic infants. It also affords an experimental method for the study of rational therapy in such cases (Keith).

The results of the present investigation are presented graphically in Charts 1 to 4 inclusive. Hemoglobin estimations are given in percentages of the normal hemoglobin values. From these graphs it is quite apparent that blood concentration changes become significant. The average increased concentration begins early in the period of water deprivation and gradually rises to a fairly well defined maximum which is not exceeded during the time interval of this investigation. The daily values for hemoglobin content fluctuate greatly which perhaps may indicate an attempt

[2] Keith, N. M., *Am. J. Physiol.*, 1922, lix, 452.

CHART 1. The influence of water introduction upon hemoglobin of the blood of animals deprived of water.

CHART 2. The influence of water introduction upon hemoglobin of the blood of animals deprived of water.

CHART 3. The influence of water introduction upon hemoglobin of the blood of animals deprived of water.

CHART 4. The influence of water introduction upon hemoglobin of the blood of animals deprived of water.

on the part of the organism to maintain constant the concentration of the circulating medium. A point of considerable significance is the maximum level of blood concentration attained. In previous experience[3] it has been shown that blood concentration exceeding 25 per cent above the normal is attended by grave symptoms and that a level maintained at 40 per cent or more is usually followed by death. In this connection it is quite evident that the results obtained in the present communication fall in line with this general statement. Levels of blood concentration considerably above 25 per cent of normal were reached but were not maintained for appreciable periods. This fact emphasizes the large factor of safety possessed by the organism in regulation of the water balance. It is quite probable therefore that grave detrimental effects are not to be expected from water deprivation until this factor of safety in water regulation has been exceeded. If the assumption is correct that levels of blood concentration exceeding 40 per cent of the normal are attended by marked evidences of abnormality then it is probable that this factor of safety breaks down when blood concentration is maintained at a level greatly exceeding 40 per cent of the normal value.

Although the fact that water deprivation appreciably increases blood concentration, is of great significance clinically, of even greater importance is the observation presented in the appended charts, that introduction of water into the system of an animal with concentrated blood will rapidly cause blood concentration to resume a level near the normal. Such observations indicate proper methods of therapy in conditions associated with highly concentrated blood. It is well known that blood concentration is altered little, if at all, by the introduction of large volumes[4] of water into normal individuals, the organism tending to maintain a constant circulating fluid. It would seem quite reasonable to assume that the introduction of large volumes of fluid into the normal organism would be promptly excreted, the excess fluid being of no special value. On the other hand, it is just as reasonable to assume that in conditions where water in the body has been greatly diminished introduced water should be retained in order to restore blood

[3] Underhill, F. P., The lethal war gases; physiology and experimental treatment, New Haven, 1920.

[4] Haldane, J. S., and Priestley, J. G., J. Physiol., 1915–16, l, 296.

volume to a normal status. Such a fact emphasizes the error of applying observations obtained under normal circumstances to conditions in which disturbed metabolic processes play a rôle. Water introduced into a normal individual will not materially alter blood concentration, introduced into an animal with highly concentrated blood water will cause blood concentration to be reduced to a level near the normal. These facts further emphasize the rational type of treatment to be followed in conditions associated with concentrated blood as already indicated for war gas poisoning[3] and certain types of influenza.[5]

CONCLUSIONS.

Short intervals of water deprivation significantly increase the blood concentration of dogs. The degree of concentration attained under the experimental conditions indicates a great factor of safety on the part of the organism in the regulation of water balance since dangerous levels of blood concentration were not maintained nor were abnormal symptoms in evidence.

Water introduction in animals with concentrated blood produces a return of blood concentration to a point approximating the normal.

These observations indicate the type of therapy adequate in conditions associated with concentrated blood and lend support to the therapy advocated previously by Underhill in war gas poisoning and in certain types of influenza.

[5] Underhill, F. P., and Ringer, M., *J. Am. Med. Assn.*, 1920, lxxv, 1531.

THE EXCRETION OF ACID AND AMMONIA.

By ROGER S. HUBBARD and SAMUEL A. MUNFORD.

(From The Clifton Springs Sanitarium, Clifton Springs, New York.)

In an investigation of the relationship between the alkaline tide in urine and the occurrence of free hydrochloric acid in the stomach as shown by fractional gastric analysis, a preliminary report of which has been given elsewhere (Hubbard and Munford, 1922), certain facts were observed regarding the excretion of ammonia which led to the present study: cases which showed an absence of free hydrochloric acid in the gastric juice showed a high degree of constancy of both hydrogen ion concentration and ammonia in specimens of urine collected at different times in the day. This observation is not new; Schittenhelm (1903), Loeb (1905), and Gammeltoft (1911) have reported similar findings in various clinical conditions associated with anacidity, and have shown that ammonia excretion varies after meals. Recently, Campbell (1920), who has summarized the earlier literature upon this subject, has shown that the excretion of acid and ammonia vary together after meals. In the paper presented here a statistical study of a series of results has been made to find out: (1) to what degree ammonia excretion varies with the acidity of the urine; (2) what influence the volume of the urine has upon the excretion of ammonia; (3) whether any relationship found is between the ammonia present in urine and the reaction or the amount of acid present; (4) is any relationship noted primarily between acidity and the amount of ammonia present, or acidity and the concentration of ammonia; and (5) what is the influence of volume of urine excreted on the relationship between acidity and ammonia.

The literature upon the alkaline tide in urine is extensive, and, as it has recently been reviewed by Campbell (1920) and Fiske (1921), it will not be summarized here. Two views have been advanced as to the cause of the alkaline tide, one that it is due to

465

the secretion of hydrochloric acid by the stomach and subsequent reabsorption of the acid in the intestines, recently restated and defended by Campbell (1920), and supported by the work of Bennett and Dodds (1921) on the relationship between gastric acidity and alveolar carbon dioxide tension, and one that it is due to changes in the respiratory activity which would lead to a condition of acidosis were they not compensated for by the secretion of a urine more alkaline than usually by the kidney (Leathes, 1919). The constancy of urine reaction found in cases showing an absence of free hydrochloric acid in the gastric juice (Hubbard and Munford, 1922) would favor a gastric rather than a renal source of the alkaline tide.

Many writers, besides those already referred to, have called attention to the relationship between reaction and ammonia excretion. Hasselbalch (1912, 1916) in particular, has discussed the simultaneous variation of these two urinary constituents after meals, and has studied the effect of differences in diet upon them. He noted that the ratio between ammonia and total nitrogen in the urine varied with the hydrogen ion concentration, and plotted curves to illustrate the relationship (Hasselbalch, 1916). In the same paper he stated that the values of this ratio corresponded more closely with those of the hydrogen ion concentration than did either the concentration of the ammonia or the amount excreted in an hour. Confirmation of the existence of a relationship between ammonia excretion and the reaction of the urine is furnished by the work of Collip and Backus (1920) and of Grant and Goldman (1920). They investigated the effect of forced respiration upon various factors which might be influenced by an induced acidosis or alkalosis, and found that the urine became less acid and the amount of ammonia excreted decreased as a result of their experiments.

Among papers which have dealt with the influence of fluid intake, with consequent increase of volume of urine excreted, upon the elimination of ammonia are those of Wills and Hawk (1914) and Wilson and Hawk (1914). These authors showed that the amount of ammonia excreted in a day varied with the amount of water taken. Ivy (1918) investigated the influence of fluid intake upon ammonia excretion, of gastric secretion upon ammonia excretion, and of fluid intake upon gastric secretion. He worked

with normal human subjects and with dogs, and showed that secretion of hydrochloric acid by the stomach and its subsequent reabsorption regularly influenced the excretion of ammonia, but that increased excretion of water did not always do so. He believed that unless water was so given that it influenced the secretion of hydrochloric acid by the stomach it had no influence upon the excretion of ammonia.

Comparisons of urinary acidity and ammonia excretion after the administration of acids and alkalies have been made often. A paper which bears directly upon some aspects of the problem under consideration is that of Marriott and Howland (1918). They fed equivalent amounts of dilute hydrochloric acid and mixtures of phosphates of different hydrogen ion concentrations to three normal human subjects, and found that the acid increased the daily elimination of both the titratable acid and ammonia, that solutions of acid sodium phosphate increased the elimination of acid only, and that phosphate solutions which had a pH of 7.4 increased the amount of acid and decreased the amount of ammonia excreted. These experiments show that titratable acid and ammonia are not necessarily related to each other. In experiments under normal conditions, however, there may be some relationship between amounts of acid and amounts of ammonia excreted. Campbell (1920) has commented upon such a relationship in his paper upon the alkaline tide, and Fiske (1920) has discussed variations in the ratio between the amounts of ammonia and sulfate excreted because sulfuric acid is one of the acids neutralized by ammonia in the body. He showed that the values of this ratio varied with the hydrogen ion concentration of urine.

The figures discussed in this paper were obtained from a series of twenty-one experiments carried out on nineteen subjects who did not show clinical or metabolic symptoms of acidosis; six of them gave gastric analyses typical of anacidity. In each experiment specimens of urine were collected every 2 hours from 7 a.m. to 7 p.m., and a single specimen was collected from 7 p.m. to 7 a.m. of the following day. All specimens were analyzed as soon as possible after they were collected, except that in some instances specimens collected in the evening were put on ice and were analyzed the next morning. The determination of the hydrogen ion concentration was carried out by a colorimetric method es-

sentially the same as that recently described[1] by Marshall (1922), the titratable acid was determined by the method of Folin (1916), and the ammonia by that of Folin and Bell (1917). Gastric analyses were made by the fractional method of Rehfuss, Bergeim, and Hawk (1914). Typical protocols of three experiments, one on a normal subject and two on cases of anacidity, are given in Table I. One of the cases of anacidity showed marked differences in the amount of urine secreted in an hour, while the volumes of the specimens obtained from the other case did not vary much.

In studying the results, it seemed best to consider each experiment separately, and not combine all the results from all the experiments, to avoid variations in the elimination of acid and ammonia by different individuals. Inspection of the data showed that there were relationships between ammonia excretion, acid excretion, and the volumes of the urine specimens, but that these relationships were not definite enough to be expressed in any simple mathematical or graphic way. The data were subjected instead to statistical analysis.

The effect of the different factors upon the excretion of ammonia and on each other was first studied in the following way. Each factor in turn was regarded as an independent variable, and the results obtained on each experiment were arranged in order of the changes in that factor. For example, all the results in each experiment were arranged in order of their volumes from highest to lowest. The total number of urines obtained in each complete experiment was seven[2] (in two of the experiments patients were unable to void at the end of 2 hours) and arranged in this way each experiment gave 21 pairs of determinations in which any one factor varied. The number of those pairs in which other factors increased or decreased with the independent variable was counted,

[1] The specimens were collected in jars which contained a small amount of toluene. No especial precautions similar to those recently described by Marshall were taken, and the results of the determinations of the hydrogen ion concentrations are subject to such corrections as have been described by him.

[2] It was thought at first that only those specimens collected at 2 hour intervals during the day should be studied, but the specimens collected during the night were found to be comparable with the others and they have been included. The results did not influence the figures appreciably except to increase the number of determinations available for comparisons.

and the result, expressed in terms of percentage of the total pairs was recorded in Table II. In this table the effect of different degrees of difference in the values of the independent variable are also shown.

TABLE 1.

Gastric.			Urine.						
Time.	0.1 N in 100 cc.		Time.	Volume per hr.	pH	Ammonia N.		0.1 N acid.	
	HCl.	Total.				mg. per 100 cc.	mg.	In 100 cc	Total.
a.m.	cc.	cc.		cc.					
Res.	0	6	7-9 a.m.	78	5.5	20.0	15.6	14.3	11.2
9.00	0	11	9-11 "	84	5.7	15.7	13.2	7.2	6.05
9.15	4	21	11-1 p.m.	99	5.9	20.0	19.8	13.2	26.1
9.30	19	35	1-3 "	66	5.3	27.8	18.4	28.9	19.1
9.45	33	48	3-5 "	91	6.1	16.9	15.3	14.1	12.3
10.00	37	69	5-7 "	44	5.45	27.8	12.1	31.2	13.6
10.15	40	81	7-7 a.m.	35	5.6	33.9	12.0	15.1	18.2
10.45	35	58							
11.15	0	10							
Res.	0	6	7-9 a.m.	39	5.1	29.0	11.3	40.0	31.9
9.30	0	10	9-11 "	40	4.9	36.5	14.4	59.1	46.6
9.45	0	5	11-1 p.m.	192	5.2	12.5	47.8	13.2	25.3
10.00	0	5	1-3 "	143	5.1	15.4	37.7	20.8	25.5
10.15	0	4	3-5 "	208	5.25	10.5	21.9	12.1	25.0
10.45	0	4	5-7 "	173	5.4	10.0	17.3	12.1	41.5
			7-7 a.m.	82	5.2	15.4	12.6	11.6	9.50
Res.	0	4	7-9 a.m.	18	5.3	51.4	9.00	26.8	4.70
9.15	0	9	9-11 "	14	5.35	44.7	6.70	31.8	8.90
9.30	0	7	11-1 p.m.	16	5.2	44.4	7.10	44.7	7.15
10.00	0	10	1-3 "	27	5.4	47.6	12.9	31.1	16.8
10.30	0	10	3-5 "	29	5.45	47.8	13.9	30.7	8.90
			5-7 "	10	5.3	56.1	5.61	37.0	3.70
			7 7 a.m.	34	5.5	50.4	15.2	18.0	6.17

Reference to the first part of Table II will make this method of analysis clear. There were 399 pairs of determinations in which the volume varied; in 74 per cent of them the hourly excretion of ammonia increased when the volume increased, and in 65 per cent the concentration of ammonia decreased as the volume increased. There were only 284 pairs which showed differences

in volume greater than 10 cc. per hour, and in 76 per cent of these the ammonia excretion varied directly as the volume, while in 71 per cent of these the ammonia concentration varied inversely as the volume.

TABLE II

Independent variable			Dependent variable				
				Ammonia		Acid	
Kind.	Variation.	Pairs	pH	Concentration.	Total.	Concentration	Total.
			per cent	per cent	per cent	per cent	per cent
Volume.	All pairs.	399	57	65	74	64	54
"	More than 10 cc. per hr.	281	62	71	76	68	53
"	" " 20 " " "	206	61	83	82	70	56
"	" " 50 " " "	86	74	86	84	69	57
Reaction.	All pairs.	365		72	59	78	72
"	More than 0.2 pH.	238		79	65	81	78
"	" " 0.5 "	130		90	77	81	86
"	" " 1.0 "	70		90	81	93	90
Acid concentration.	All pairs.	387	74	75	55		
" "	More than 10 per cent.	353	74	79	54		
" "	" " 25 " "	282	83	79	54		
" "	" " 50 " "	161	85	87	45		
Total acid.	All pairs.	398	66	64	73		
" "	More than 10 per cent.	353	64	66	72		
" "	" " 25 " "	257	72	70	77		
" "	" " 50 " "	116	76	68	85		

All dependent variables vary directly with the independent ones except the concentrations of ammonia and acid, which vary inversely as the volume. Figures for hydrogen ion concentration compared with volumes show actual increases in the hydrogen ion concentration as the volume decreases. Ammonia and acid excretion and concentration increase as the hydrogen ion concentration increases.

The first part of Table II shows: (1) that urine ammonia, whether expressed as concentration or rate of elimination, varies with the volume eliminated; (2) that the amount of acid eliminated

does not vary with the volume; (3) that the hydrogen ion concentration of urine has a slight tendency to increase as the volume decreases, but that the differences are so slight that it is not safe to draw any conclusions from the figures. The second part of the table shows that variations in both acid and ammonia elimination whether expressed as concentrations or as amounts excreted, vary with the hydrogen ion concentration, but that there is better agreement between the reaction of urine and the concentration of ammonia than between the reaction and the amounts of ammonia eliminated. The third and fourth parts of the table show that differences in the ammonia and acid elimination agree with each other, but that they do not agree better than would be expected from the relationship both show to the hydrogen

TABLE III.

Reactions vary 0.2 pH or less.

Variable.	Difference.	No.	Amount of ammonia.	Amount of acid.
			per cent	per cent
Volume.	All pairs.	153	89	71
"	More than 10 cc.	93	94	76
"	" " 20 "	60	97	75
Acid.	All pairs.	150	77	
"	More than 10 per cent.	124	86	
"	" " 20 " "	78	85	

ion concentration of urine. The most striking fact shown by the table is the slight effect which changes in volume have upon the amount of acid excreted.

In Table III are recorded variations in the amounts of ammonia and of acid eliminated when the hydrogen ion concentration is constant within 0.2 pH (this represents the probable limit of accuracy of the determination on samples of urine collected as these were (Marshall, 1922)) and the volume varies. The table shows that, under such conditions the rate of ammonia excretion varies (qualitatively) almost directly with the volume, but that the rate of acid excretion does not. The table also shows that, when the reaction of the urine is constant, the excretion of ammonia varies more closely with the volume than it does with the amount of acid excreted.

Table IV shows the results of studies upon the concentration of acid and ammonia when the volumes of the samples did not differ from each other by more than 20 cc. Both vary to about an equal degree with the reactions of the specimens, but there is no better agreement between the ammonia concentration and the acid concentration than there is between the ammonia concentration and the reaction. A similar table to the one given was constructed based on the pairs which showed agreement within 10 cc. and the figures were essentially the same as those given in Table IV. From the studies discussed above it is evident that the ammonia excretion varies with the volume and reaction, but not with the amount of acid excreted; this corresponds with the experimental results of Marriott and Howland (1918).

TABLE IV.

Volumes vary 20 cc. or less.

Variable.	Difference.	No.	Concentration of ammonia.	Concentration of acid.
			per cent	per cent
Reaction.	All pairs.	172	65	64
"	More than 0.2 pH.	91	75	79
"	" " 0.5 "	41	96	90
Acid concentration.	All pairs.	192	67	
" "	More than 10 per cent.	163	70	
" "	" " 25 " "	121	77	
" "	" " 50 " "	60	85	

In Table V conditions under which ammonia and acid excretion are constant are given. In each experiment the number of pairs which agreed with each other within 10 and 25 per cent were counted, and the results compared with increasing constancy of volume and hydrogen ion concentration of the specimens. That changes in acid excretion and ammonia excretion are not directly related to each other is shown in this table, for, while the constancy of ammonia increases as the constancy of volume increases, that of acid does not; and while the constancy of both acid and ammonia concentrations increase as differences in reaction become less, the increases are distinctly more marked in the case of the ammonia concentrations.

The data presented in Table V furnish an opportunity for comparing the relationship of volume and reaction to concentration and rate of ammonia excretion, respectively. The table shows that the constancy of both increases as differences in volume decrease, and that constancy of concentration increases more as the volume diminishes than does the constancy of the amount of ammonia eliminated. Decreasing differences in the hydrogen ion concentration of urine is accompanied by little if any change in the constancy of the amount of ammonia eliminated, but by a great increase in the constancy of the concentration at which it is eliminated.

TABLE V.

Total pairs.			Ammonia.				Acid.			
Description.	No		Mg. per 100 cc. within		Mg. per hr. within		Mg. per 100 cc. within		Mg. per hr. within	
			10 per cent	25 per cent	10 per cent	25 per cent	10 per cent	25 per cent	10 per cent	25 per cent
			per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent
All pairs.	406		26	43	14	40	13	32	15	37
Within 0.5 pH.	273		31	57	13	46	14	37	13	46
" 0.2 "	153		35	69	12	48	20	48	20	45
All pairs.	406		26	43	14	40	13	32	15	37
Within 20 cc.	187		35	61	17	48	16	38	18	43
" 10 "	127		36	70	20	58	13	42	20	41

In Table VI the constancy of ammonia concentration and excretion when volume and reaction do not vary is studied further. In the first part of the table all pairs which showed variations of volume less than 10 cc. are subdivided into those the hydrogen ion concentration of which varies by 0.2 pH or less, by 0.5 pH or less, and by 1.0 pH or less. In the second part all pairs which showed variations of hydrogen ion concentration of 0.2 pH or less are divided in a similar way according to the constancies of their volumes. The table shows the following relationships between the independent variables, constancy of volume and reaction, and the dependent variables, constancy of ammonia concentration and excretion; there is greater constancy of ammonia concentration than of ammonia excretion throughout both parts

of the table, even when both the reaction and the volume are constant; constancy of volume without constancy of reaction is accompanied by greater constancy in the amount of ammonia excreted than is the opposite condition; constancy of ammonia concentration varies more nearly with the reaction than with the volume, for if the hydrogen ion concentration is constant, only rather marked changes in volume variation are accompanied by significant changes in the constancy of ammonia concentration. (See figures for volumes within 10 and 20 cc. in the table showing pairs having hydrogen ion concentrations within 0.2 pH.)

TABLE VI

All Cases.

Description of pairs			Ammonia.			
Variable.	Difference.	No.	Mg. per 100 cc. within		Mg. per hr. within	
			10 per cent	25 per cent	10 per cent	25 per cent
			per cent	*per cent*	*per cent*	*per cent*
Volume within 10 cc.	All pairs.	127	36	70	20	58
" " 10 "	pH in 1.0.	115	39	73	21	61
" " 10 "	" " 0.5.	100	44	80	22	65
" " 10 "	" " 0.2.	65	41	89	22	78
Within 0.2 pH.	All pairs.	153	35	69	12	48
" 0.2 "	Volume in 100 cc.	142	38	73	13	49
" 0.2 "	" " 50 "	127	41	80	13	55
" 0.2 "	" " 20 "	85	46	88	18	69
" 0.2 "	" " 10 "	65	41	89	22	78

The values of the hydrogen ion concentration and of other factors may change during the periods which correspond to the collection of samples of urine. Cases of gastric anacidity show a high degree of constancy in the reaction of different samples of urine, and probably do not show such great changes during the periods of collection as do other types of cases. Material from cases showing gastric anacidity should show a closer correspondence between reaction and any constituent which varies with reaction than does material from unassorted cases, and tables paralleling those given have been prepared from the data obtained

from the six cases of anacidity studied. Table VII was prepared in the same way as was Table VI, but contains determinations on these six cases only. This table shows the same relationships which were shown by the preceding table, but shows them present in a more marked degree. Ammonia concentration shows great constancy when the hydrogen ion concentration is constant, and only large differences in volume (more than 50 cc. per hour) markedly reduce this constancy. Ammonia concentration also increases in constancy as the volume differences become smaller, even when the reaction remains constant; this change is not as

TABLE VII.

Cases of Anacidity.

Description of pairs.			Ammonia.			
			Mg. per 100 cc. within		Mg. per hr. within	
Variable.	Description.	No.	10 per cent	25 per cent	10 per cent	25 per cent
			per cent	per cent	per cent	per cent
Volume within 10 cc.	All pairs.	56	48	86	23	68
" " 10 "	pH in 0.5.	52	58	92	25	69
" " 10 "	" " 0.2.	38	61	97	22	82
Within 0.2 pH.	All pairs.	78	41	81	10	50
" 0.2 "	Volume in 100 cc.	71	45	87	13	55
" 0.2 "	" " 50 "	62	52	97	15	61
" 0.2 "	" " 20 "	50	52	98	18	70
" 0.2 "	" " 10 "	38	61	97	22	82

marked as the other, however, and, as a matter of fact, no pairs agreed within 25 per cent when the difference in hydrogen ion concentration was more than 0.5 pH, and none agreed which showed a difference of more than 0.3 pH, except when the volumes of the specimens varied 10 cc. or less. A comparison of the figures for ammonia concentration with those for the amount of ammonia excreted, shows clearly that the constancy of ammonia excreted does not primarily vary with the reaction, for there is good agreement with the hydrogen ion concentration only when the volumes of urine are also equal, and even then there is not as good agreement of the values as there is of the values of the ammonia con-

centration. The equality under these conditions—like concentrations and like volumes—is inevitable.

The relationship between hydrogen ion concentration and volume on the one hand, and ammonia excretion on the other, is

CHART 1. Equal amounts of ammonia excreted.

shown in the charts. In Chart 1 the volume differences and hydrogen ion differences of each pair of determinations (from all the cases studied, not from the cases of anacidity alone) which showed an agreement of the amount of ammonia excreted within

10 per cent is shown; Chart 2 gives the corresponding values
for pairs which showed the same constancy of ammonia concen-

CHART 2. Equal concentrations of ammonia.

tration.   Chart 2 shows clearly that there is a definite relation-
ship between the volume and hydrogen ion concentration on the one

hand, and the constancy of the concentration of ammonia on the other, while Chart 1 shows that there is no such relationship of constancy of ammonia excreted except when both volume and reaction are constant.

Study of the tables and charts shows: (1) that ammonia excretion varied with the acidity of the urine; (2) that ammonia excretion varied with the volume of the urine excreted; (3) that the excretion of ammonia varied with the reaction of the urine, and that the amount of acid excreted did not directly affect its excretion; (4) that the agreement between changes of reaction and ammonia excretion was primarily between hydrogen ion concentration and the concentration of ammonia, not between hydrogen ion concentration and the amount of ammonia excreted; and (5) that large differences in volume led to a diminished agreement between the reaction and the concentration of the ammonia, while very small differences in volume sometimes were accompanied by concentrations which agreed more than the differences in the reaction would have indicated.

Do the results presented accord better with the theory recently discussed by Nash and Benedict (1921) that the kidney itself makes the ammonia which it excretes, or with the theory that it is produced elsewhere in the body? Explanations of the findings could probably be made based upon either theory, but the fact that it is the ammonia concentration, rather than the amount of ammonia excreted which varies with the reaction of the urine, appears to favor the theory that it is the kidney which is the source of ammonia. If the ammonia were produced elsewhere under the same stimulus which produces variations in the reaction of urine, a correspondence between that stimulus and the amount of ammonia produced would be expected, and a correspondence (modified to some extent by changes in the hourly rate of urine excretion) between the hydrogen ion concentration and the amount of ammonia excreted would result. Instead, there is agreement primarily between this stimulus (measured by the reaction of the urine) and the concentration of ammonia, modified only when the differences in volume are very large or very small. Since ammonia is much more concentrated in the urine than it is in the blood, and so cannot be classed with those diffusible compounds which show close agreement between the concentrations in blood

and urine (Widmark, 1920) this agreement between hydrogen
ion concentration and ammonia concentration seems to correspond
with what would be expected if the kidney produced ammonia
rather than with theories that it is produced elsewhere in the or-
ganism. No matter which theory of ammonia formation is
accepted, the facts presented in this paper tend to show that if
the volume of the urine can be increased in some way which does
not lead to changes in the hydrogen ion concentration of the urine,
or rather of the factors which control the hydrogen ion concentra-
tion of the urine (ingestion of water may do so by inducing a
secretion of gastric juice (Bergeim, Rehfuss, and Hawk, 1914))
the amount of ammonia eliminated will be increased.

BIBLIOGRAPHY.

Bennett, T. I., and Dodds, E. C., *Brit. J. Exp. Path.*, 1921, ii, 58.
Bergeim, O., Rehfuss, M. E., and Hawk, P. B., *J. Biol. Chem.*, 1914, xix, 345.
Campbell, J. A., *Biochem. J.*, 1920, xiv, 603.
Collip, J. B., and Backus, P. L., *Am. J. Physiol.*, 1920, li, 568,
Fiske, C. H., *J. Biol. Chem.*, 1920, xli, p. xxxix.
Fiske, C. H., *J. Biol. Chem.*, 1921, xlix, 163.
Folin, O., Laboratory manual of biological chemistry, New York and Lon-
    don, 1916, 103.
Folin, O., and Bell, R. D., *J. Biol. Chem.*, 1917, xxix, 329.
Gammeltoft, S. A., *Z. physiol. Chem.*, 1911, lxxv, 57.
Grant, S. B., and Goldman, A., *Am. J. Physiol.*, 1920, lii, 209.
Hasselbalch, K. A., *Biochem. Z.*, 1912, xlvi, 403.
Hasselbalch, K. A., *Biochem. Z.*, 1916, lxxiv, 18.
Hubbard, R. S., and Munford, S. A., *Proc. Soc. Exp. Biol. and Med.*, 1921-
    22, xix, 429.
Ivy, A. C., *Am. J. Physiol.*, 1918, xlvi, 340.
Leathes, J. B., *Brit. Med. J.*, 1919, ii, 165.
Loeb, A., *Z. klin. Med.*, 1905, lvi, 100.
Marriott, W. McK., and Howland, J., *Arch. Int. Med.*, 1918, xxii, 477.
Marshall, E. K., Jr., *J. Biol. Chem.*, 1922, li, 3.
Nash, T. P., Jr., and Benedict, S. R., *J. Biol. Chem.*, 1921, xlviii, 463.
Rehfuss, M. E., Bergeim, O., and Hawk, P. B., *J. Am. Med. Assn.*, 1914,
    lxiii, 909.
Schittenhelm, A., *Deutsch. Arch. klin. Med.*, 1903, lxxvii, 517.
Widmark, E. M. P., *Biochem. J.*, 1920, xiv, 364.
Wills, F., and Hawk, P. B., *J. Am. Chem. Soc.*, 1914, xxxvi, 158.
Wilson, D. W., and Hawk, P. B., *J. Am. Chem. Soc.*, 1914, xxxvi, 1774.

STUDIES OF GAS AND ELECTROLYTE EQUILIBRIA IN THE BLOOD.

III. THE ALKALI-BINDING AND BUFFER VALUES OF OXYHEMO-GLOBIN AND REDUCED HEMOGLOBIN.

By DONALD D. VAN SLYKE, A. BAIRD HASTINGS, MICHAEL
HEIDELBERGER, AND JAMES M. NEILL.

(From the Hospital of The Rockefeller Institute for Medical Research.)

The general problem of which the present paper attempts to solve a portion has been outlined in the first of these studies (1). The objects of the present work have been to determine quantitatively, on solutions of hemoglobin free from other proteins, the following data.

1. The amounts of base bound by oxyhemoglobin and reduced hemoglobin, respectively, at normal blood pH.

2. The changes in the base bound by oxyhemoglobin and reduced hemoglobin with changing pH (the buffer value, $\dfrac{dB}{dpH}$, in the sense outlined by Van Slyke (2)).

3. The nature of the curve relating oxygen increase to the increase in base bound at constant pH that occurs as reduced hemoglobin adds varying amounts of oxygen.

The unsatisfactory state of the present knowledge of the base-binding and buffer values of hemoglobin may be seen by referring to previous theoretical papers (2, 3) in which data for only the roughest approximate estimations were found available.

The increase in blood of the CO_2-replacing power (which is equivalent to increase in base-binding power) of hemoglobin when changed from the reduced to the oxygenated form was discovered by Christiansen, Douglas, and Haldane (4) and its physiological significance in the CO_2-carrying power of the blood was clearly pointed out. These authors published a single pair of curves, one of oxygenated, the other of reduced blood, obtained

by saturating samples of blood drawn at different times from the same individual. The blood was analyzed by methods which, as pointed out in our first paper (1), like those used by ourselves until the requirements of the present work forced the development of refinements, were adequate only to give approximate knowledge of the magnitude of the changes involving as a maximum 6 or 7 volumes per cent of CO_2. Up to the present the single pair of curves, published by Christiansen, Douglas, and Haldane and a similar pair published by Joffe and Poulton (5) have remained almost the entire experimental basis of knowledge concerning the mutual effects of oxygen and carbon dioxide tensions in the blood.

Concerning the nature of the intermediate relationships (*i.e.* whether the first portion of oxygen taken up by reduced hemoglobin has more or less effect on its base-binding power than the last portion), Christiansen, Douglas, and Haldane have tacitly assumed, in their calculations explaining the physiological significance of the phenomenon, that the change is a linear one, the first portion of oxygen taken up having the same relative effect in CO_2 replacement as the last. The same assumption has been made by L. J. Henderson (6), Van Slyke (3), and Peters, Barr, and Rule (7), although admittedly without experimental basis.

The assumption is in fact an essential part of Henderson's theory explaining the O_2-CO_2 effect on hemoglobin by assuming that the oxygenated form is an acid, $HHbO_2$, with a greater dissociation constant than the reduced form, HHb. If this is the case the proportion of alkali bound by each form is, at a given pH, fixed in accordance with the equation $H^+ = K'_O \dfrac{[HHbO_2]}{[BHbO_2]}$ and $H^+ = K'_R \dfrac{[HHb]}{[BHb]}$, K_O and K_R representing the dissociation constants of the carboxyl groups of oxygenated and reduced hemoglobin affected by the change in oxygenation. Under these conditions, at a given pH, the alkali bound by hemoglobin would change in exact proportion to the oxygen taken up, the ratio $\dfrac{dB}{d[HbO_2]}$ at a given pH being constant.

The data in the present paper confirm the above assumptions as to the straight line form of the curve relating changes in oxygen to changes in base bound by hemoglobin.

We have found that satisfactory settlement of the questions outlined in the introduction, but especially of the third, demanded technique of a different order of accuracy from that available when the problem was at first attempted. Finally with the development of Heidelberger's method (8) for preparing readily and in large amounts crystalline hemoglobin practically electrolyte-free, with the attainment of an accuracy of about 1 part in 500 in blood gas analyses by means of the "constant volume" apparatus (9), and with the evolution of the general technique for saturation and handling of blood and hemoglobin solutions outlined in the first paper, especially the use of the double-chambered tonometer described there under the "first saturation method," our technique became refined in the necessary points to such a degree that reproducible quantitative results could be obtained. It will be seen that the added errors of manipulation and of the three analyses involved, $viz.$, blood O_2, blood CO_2, and CO_2 tension in the final gas mixtures, cause deviations on the average of not over 0.1 mM. of $BHCO_3$ (0.2 volume per cent of CO_2), from the mean linear curve, although each experiment shows one or two points with somewhat wider deviation.

<center>EXPERIMENTAL.</center>

The hemoglobin crystals were prepared by Heidelberger's (8) method, and used as soon as possible after they had been twice recrystallized. They were dissolved in water to which NaOH was added to make the Na concentration indicated in the tables, 0.04 N in Experiment 1, 0.03 N in Experiments 2, 3, and 4. It will be seen from the table headings that the preparations all fell somewhat short of 100 per cent of their theoretical oxygen capacity. This is not due to admixture of other substances, but to the extreme readiness of hemoglobin to tautomerize in some manner and lose some of its ability to bind labile oxygen. If dried, even at high vacuum and in the cold, hemoglobin is almost completely inactivated, although in solubility and color it still resembles oxyhemoglobin. Slow loss of activity occurs even while standing in solution. The maximum loss in our preparations was one-fifth of the theoretical oxygen capacity. There is no reason to expect such inactivation of part of the hemoglobin to affect the alteration of the rest in base-binding power when oxy-

gen is added or lost. The presence of inactive hemoglobin in our preparations, however, increases in a way discussed later, the limit of error that must be allowed in estimating the absolute amounts of base bound per mol of oxyhemoglobin and reduced hemoglobin.

The hemoglobin was dissolved to make about the same concentration found in horse blood, about 7 mM. (equivalent to 15 to 16 volumes per cent of oxygen capacity), and the amount of alkali added was such that the system at physiologically normal CO_2 tensions had pH values within physiological limits.

The saturations were performed at 38° with the double tonometer elsewhere described (1). Usually 20 or 30 cc. of solution were saturated with 300 cc. of gas. The lower chamber of the tonometer was filled with solution and clamped off from the upper. When low oxygen tensions were desired the upper chamber was evacuated with a water pump and washed out three times with hydrogen before the final gas mixture was let in. After 20 minutes saturation the gas mixture was renewed, in order to make certain that the final conditions should approximate as closely as possible those desired. After preliminary experiments the behavior of the hemoglobin was known well enough to permit rather close prediction of the pH, [BHCO₃], and [HbO₂] values that would be obtained.

One of our main purposes was to ascertain the change in alkali bound when the oxygen saturation of the hemoglobin was varied, the pH being kept constant. It was impracticable so closely to control conditions that a series of solutions with varying oxygen and CO_2 tensions should all be brought to exactly the same pH, although it could be approximated. Having determined the base bound by the hemoglobin at the pH obtained, say 7.27 instead of a desired 7.30, the base bound at 7.30 was estimated by extrapolation. This was performed by determining the slope of the B, pH line (B representing the total base bound by hemoglobin), by means of several points on completely oxygenated solutions with varying CO_2 tensions. The slope, as shown in the following paper, increases with increasing oxygenation, but the change is small enough to permit neglecting it for short distance extrapolations. Consequently, lines were drawn through the reduced and intermediate points parallel to the line on which

the oxygenated points lay, and the intersections of these lines with the desired pH line were used to estimate the base bound at that pH by hemoglobin oxygenated to the extent ascertained. Examples of this mode of extrapolation are seen in Figs. 2a, 3a, and 4a.

From the base bound by hemoglobin thus ascertained at a given pH with varying degrees of oxygen saturation a curve was drawn showing the relation of oxygen content to the base bound at the given pH (Figs. 2b, 3b, 4b).

The CO_2 tensions were determined as described in connection with the saturation method (1) by analysis by Haldane's method of the separated gases in the upper chamber.

The CO_2 contents of the solutions were determined by means of the "constant volume" apparatus (9). The apparatus used had a total volume of 100 cc. The gas was measured at 5 cc. volume, which could be read with an error not exceeding 1 part per 1,000. Samples of either 2 or 3 cc. of solutions were taken, and the changes in pressure read were in the neighborhood of 100 to 160 mm. with 2 cc., and 150 to 240 mm. with 3 cc. samples. The accuracy with which duplicates could be obtained may be indicated by the following series, obtained in the course of Experiment 3 by Neill. Expressed in millimols of total CO_2 the results were:

Saturation 1, 15.40 and 15.31; Saturation 2, 20.17 and 20.12; Saturation 3, 22.54 and 22.47; Saturation 4, 22.07 and 22.02; Saturation 5, 19.12 and 19.10; Saturation 6, 19.10 and 19.08; Saturation 7, 18.80 and 18.70; Saturation 8, 18.93, 18.73, and 18.76; Saturation 9, 21.50 and 21.45; and Saturation 10, 21.07 and 21.11. In the above series 2 cc. samples were used. When duplicates failed to agree within 0.1 mm. as in Saturation 8, a third sample was analyzed. Excluding the one obviously faulty first analysis of No. 8, the average difference between duplicates was 0.05 mm. Later when 3 cc. samples were used, the average error fell to 0.03 mm. which is about 1 part in 500 of the values determined.

For the oxygen determination in the solutions an apparatus with a total volume of 50 cc. was used. The pressures were measured with the gas volume at 2.0 cc. for oxygenated blood, and at 0.5 cc. for blood near complete reduction. The oxygen was de-

termined from the change in pressure before and after absorption with pyrogallol. The maximum deviation of duplicates accepted was 0.1 mM., but analyses were usually repeated if 0.05 mM. was exceeded.

The maximum difference accepted in the duplicate Haldane analyses of the gas phase was 0.04 per cent.

If the difference between duplicates exceeded the values indicated as acceptable, and other analyses could not be done, the results of both duplicates are given in the table.

In calculating pH, if an error of 1 part per 100 occurred in the Haldane determination of the CO_2 tension in the gas and of 1 part per 200 in the CO_2 determination in the solution, and both errors were in the opposite direction, their combined effect on the pH would be a change of 0.007, which may be taken as the maximum error assignable to the estimations of pH changes. Our curves indicate that the actual error was usually less.

The sets of analyses in each table are numbered in the order in which the solutions were saturated.

Symbols Used.

The following, for the most part already familiar, symbols are used. $[BHCO_3]$, $[H_2CO_3]$, $[CO_2]$ for concentration of $NaHCO_3$, H_2CO_3, and total CO_2, respectively, in the solutions; $[O_2]$ for the total oxygen concentration; $[HbO_2]$ for the concentration of oxygen combined with hemoglobin; $[Hb]$ for the concentration of reduced hemoglobin; C for the concentration of total hemoglobin in all forms; $[BHbO_2]$ and $[BHb]$ for concentrations of univalent base bound by oxyhemoglobin and reduced hemoglobin, respectively; B for the total base bound in both forms. All these concentrations are expressed in millimolar (mM.) terms, the molecular concentrations of C, HbO_2, and Hb being taken equal to that of the maximum oxygen with which the hemoglobin is combined or could combine.

The carbon dioxide and oxygen tensions in mm. of mercury are expressed as p_{O_2} and p_{CO_2}.

The molecular buffer values of oxyhemoglobin and reduced hemoglobin are indicated as β_O and β_R, respectively.

The value calculated for the molecular buffer effect is independent of the concentration unit used, as long as the same unit is used for both B and C in the ratio $\frac{dB}{CdpH}$. In this paper we have for convenience expressed concentrations in millimolecular instead of molecular units, but the β_0 values are the same as when M is the unit of concentration. This is not, of course, true of the $\frac{dB}{dpH}$ ratio, which when expressed in millimolar terms, as in this paper, is 1,000 times as great as when expressed as molar units. A $\frac{dB}{dpH}$ blood value of 20 in this paper therefore corresponds to one of 0.020 in Van Slyke's paper on buffers (2).

Calculations.

The calculation of $[BHCO_3]$ from the total $[CO_2]$ and the p_{CO_2} was according to Formula 2, of Table IV, of our first paper (1). The calculation of pH from CO_2 and p_{CO_2} was made according to Formula 3 of the same table. The total base bound by hemoglobin, $[BHbO_2] + [BHb]$, was obtained by subtracting the $[BHCO_3]$ from the total base added. For example, in Table III, the total base added was 30.00 mM. The $[BHCO_3]$ in Solution 1 was 14.49. Hence the remainder of base, bound by the hemoglobin (since no other acids were present), was $30.00 - 14.49 = 15.51$.

Constants.

The value of pK' in the equation $pH = pK' + \log \frac{[BHCO_3]}{[H_2CO_3]}$ was determined experimentally on several hemoglobin solutions by determining in reduced solutions the $[H_2CO_3]$ and $[BHCO_3]$ as above described, and the pH electrometrically. The results given in Table V, indicate, as the average value, pK' = 6.18.

The solubility coefficient for CO_2, $\alpha_{CO_2} = 0.531$ (given in Table V, first paper (1)), was estimated as follows: Blood, plasma, and hemoglobin were acidified to pH 4.3 with HCl and the CO_2 content was determined after saturating with pure CO_2 at atmos-

pheric tension. The determinations were made on 5 cc. samples, which were transferred to the constant volume apparatus without exposure to air. The error was about 1 part in 500, and we obtained theoretical results for α_{CO_2} in pure water. For blood and plasma we found at $38°$ α_{CO_2} values 0.501 and 0.531, respectively. Each was 0.10 below Bohr's (10) estimate made on

TABLE 1.

Crystalline Horse Hemoglobin No. 5.

Total hemoglobin content of solution.....7.97 mM. Colorimetric.
Oxygen capacity of solution.............6.89 "

$$\frac{\text{Oxygen capacity}}{\text{Total hemoglobin content}} \quad \ldots\ldots\ldots\ldots\ldots 0.854$$

Conductivity of saturated HbO_2 solution in H_2O..4.3×10^{-5} mhos.
Concentration of $NaHCO_3$ added to solution.............40 mM.

Equilibration Data.

No.	P_{CO_2}	Total [CO_2].	[BHCO$_3$]	[BHbO$_2$]	pH
	mm.	*mM.*	*mM.*	*mM.*	
1	23.3	20.75	20.02	19.98	7.541
2	26.3	21.40	20.58	19.42	7.50
3	31.0	22.58	21.61	18.39	7.45
4	42.4	24.87	23.55	16.45	7.35
5	41.6	24.80	23.50	16.50	7.358
6	46.4	25.66	24.21	15.79	7.325
7	54.1	26.95	25.27	14.73	7.276
8	55.9	27.31	25.57	14.43	7.268
9	61.1	28.00	26.10	13.90	7.237
10	101.0	32.17	29.03	10.97	7.066

the assumption that hydrogen and CO_2 have the same relative solubilities in blood as in water. We have reason to believe that Bohr's estimate was close, and that our method, perhaps because of hydration of the acidified proteins, gives slightly too low results. With 7 mM. hemoglobin plus NaOH to 0.03 N concentration we found by our method an α_{CO_2} of 0.521. Adding the 0.10 correction, apparently inherent in our method, gives 0.531.

EXPLANATION OF FIGURES.

In Figs. 1, 2a, 3a, and 4a the relations between pH and base B bound by oxyhemoglobin are shown in each case by the slope

FIG. 1. From the line through the oxygenated points 1 to 9, $\frac{dB}{dpH}$ is calculated as 23.0, and β_0 as 2.58.

of the line through the three or more points representing oxygenated solutions. The molecular buffer effect β_0 is calculated as $\frac{dB}{CdpH}$, where $\frac{dB}{dpH}$ is the slope of the line through the oxy-

genated points, and C is the concentration of hemoglobin in the solution.

In Figs. 2a, 3a, and 4a the effect of varying degrees of oxygenation on the base bound by hemoglobin at constant pH is shown by the levels at which lines through points representing oxygenated, reduced, and partially reduced solutions cut a given pH

TABLE II.

Crystalline Horse Hemoglobin No. 7.

Total hemoglobin content of solution.....6.71   mM. Colorimetric.
                                          7.13   "   Kjeldahl.
                                          6.92   "   Mean.
Oxygen capacity of solution..............5.50   "

$$\frac{\text{Oxygen capacity}}{\text{Total hemoglobin content}} \ldots \ldots \ldots \ldots 0.795$$

Conductivity of saturated HbO_2 solution in H_2O..3.8×10^{-5} mhos.
Concentration of NaOH added to solution...............30 mM.

Equilibration Data.

No.	P_{O_2}	Total [O_2].	[HbO_2].	P_{CO_2}	Total [CO_2].	[$BHCO_3$]	[BHb] + [$BHbO_2$]	pH	[BHb] + [$BHbO_2$] at pH 7.30.
	mm.	mM.	mM.	mm.	mM.	mM.	mM.		mM.
1	143.1	5.52	5.33	29.5	16.20	15.28	14.72	7.403	⎫ 12.80
2	137.5	5.25	5.07	57.3	20.72	18.94	11.06	7.207	⎬
3	131.8	5.18	5.01	84.3	24.01	21.39	8.61	7.092	⎭
5*	10.0	1.74	1.73	43.7	20.48	19.12	10.88	7.328	10.28
6	20.0	3.08	3.05	44.1	20.13	18.75	11.25	7.315	10.95
7	34.5	4.12	4.08	45.0	19.55	18.15	11.85	7.292	11.93
					19.66	18.26	11.74	7.295	
8	28.4	3.86	3.83	57.4	21.55	19.76	10.34	7.224	11.75
9	5.7	1.05	1.04	43.2	20.78	19.43	10.57	7.340	9.70

* No. 4 was lost.

line. The values indicated by these intersections are given in the last columns of Tables II, III, and IV.

In Figs. 2b, 3b, and 4b, the amounts of base indicated by the above described intersections are plotted against amounts of oxygen combined with the hemoglobin. The curves in all three cases are straight lines. The values of the molecular increments

of base added by the hemoglobin at the given pH per molecular increment of oxygen are indicated as $\dfrac{dB}{d[HbO_2]}$.

Fig. 2 a. From the line through the oxygenated points 1, 2, and 3, $\dfrac{dB}{dpH}$ is calculated as 18.4, and β_0 as 2.66.

Intersections of lines through the other points with the pH 7.30 line indicate effects of varying degrees of reduction in lowering the amount of base bound by the hemoglobin.

The numbers near the points indicate the data in Table II from which they are taken. In the last column of the table the amounts of base indicated by the intersections on the pH 7.30 line are given.

DISCUSSION OF RESULTS.

It is convenient to discuss here in their reverse order the three questions put at the beginning of this paper.

The Nature of the Curve Relating Increment of Oxygen Bound to Increment of Base Bound by Hemoglobin at Constant pH.

As shown by Figs. 2b, 3b, and 4b, the $\dfrac{dB}{d[HbO_2]}$ curve is a straight line.

FIG. 2 b. Amounts of base bound by hemoglobin at pH 7.30 compared with amounts of oxygen bound. Data from Table II and Fig. 2 a.

From the slope of the line, $\dfrac{dB}{d[HbO_2]}$ is calculated as 0.74.

The Buffer Value of Oxyhemoglobin over the Physiological pH Range, and the Indicated Minimum Number of Monovalent Acid Groups Active as Buffers at That Range.

a. Oxyhemoglobin.—By Figs. 1, 2a, 3a, and 4a the B, pH lines for oxygenated hemoglobin solutions are shown to be straight over at least the range pH 7.2 to 7.5. The values of the molecular buffer ratios were $\beta_O = 2.58$, 2.66, 2.68, and 2.63, average 2.64.

It has been previously shown (2) that the maximum molecular buffer value, β_M, of one monovalent acid group is 0.575. The minimum number of acid groups in a polyvalent acid that are acting as buffers at a given pH is therefore indicated by dividing

TABLE III.

Crystalline Horse Hemoglobin No. 11.

Total hemoglobin content of solution	.....7.73	mM.	Colorimetric.
	7.54	"	Kjeldahl.
	7.64	"	Mean.

Oxygen capacity of solution..............7.13   "

$$\frac{\text{Oxygen capacity}}{\text{Total hemoglobin content}}...............0.933$$

Conductivity of saturated HbO_2 solution in H_2O..3.8×10^{-5} mhos.
Concentration of NaOH added to solution................30 mM.

Equilibration Data.

No.	P_{O_2}	Total $[O_2]$	$[HbO_2]$	P_{CO_2}	Total $[CO_2]$	$[BHCO_3]$	$[BHb] + [BHbO_2]$	pH	$[BHb] + [BHbO_2]$ at pH 7.31
	mm.	mM.	mM.	mm.	mM.	mM.	mM.		mM.
1	144.2	7.13	6.94	28.0	15.36	14.49	15.51	7.401	
2	138.7	7.18	7.00	55.0	20.15	18.44	11.56	7.212	
				55.8		18.41	11.59	7.205	13.63
3	135.0	6.96	6.78	72.9	22.51	20.24	9.76	7.130	
				73.4		20.23	9.77	7.127	
4	3.2	0.60	0.60	48.9	22.05	20.53	9.47	7.310	9.47
5	14.8	3.10	3.08	38.6	19.11	17.19	12.09	7.354	11.15
6		4.51	4.48						
	22.6	4.74	4.71	42.9	19.09	17.75	12.25	7.304	12.35
7	20.1	3.99	3.96	39.6	18.75	17.52	12.48	7.333	12.00
8	23.9	4.77	4.74	41.5	18.75	17.46	12.54	7.311	12.50
9	4.2	0.84	0.83	45.9	21.48	20.05	9.95	7.327	9.57
10	8.8	1.54	1.53	45.9	21.09	19.66	10.34	7.319	10.12

the observed β_M value by 0.575. If we do this with oxyhemoglobin we obtain $\frac{2.64}{0.575} = 4.6$. It is evident that at least five buffer groups in the hemoglobin molecule are active over this range.

By "active" is meant that they are partially, but only partially, combined with alkali. The maximum buffer effect is exerted when a buffer acid is combined with half an equivalent of base (2). If there were five groups, each exerting maximum buffer value at a given pH, they would bind altogether 2.5 molecules

FIG. 3 a. From the line through the oxygenated points 1, 2, and 3, $\frac{dB}{dpH}$ is calculated as 20.2, and β_0 as 2.68.

Intersections of lines through the other points with the pH 7.31 line indicate effects of varying degrees of reduction in lowering the amount of base bound by the hemoglobin.

The numbers near the points indicate the data in Table III from which they are taken. In the last column of the table the amounts of base indicated by the intersections on the pH 7.31 line are given.

of univalent alkali. As a matter of fact it will be shown that
at pH 7.4 oxyhemoglobin does bind 2.1 equivalents of alkali.
So it seems fairly probable that six or seven acid groups may be
acting. That there are less than five is excluded. It is obvious
that with the alkali distributed among five or more acid groups
in the hemoglobin molecule it is impossible to calculate a single
dissociation constant in the manner applicable to a monovalent
acid.

FIG. 3 b. Amount of base bound by hemoglobin at pH 7.31 compared
with amounts of oxygen bound. Data from Table III and Fig. 3 a.

From the slope of the line, $\dfrac{dB}{d[HbO_2]}$ is calculated as 0.69.

b. *Buffer Value of Reduced Hemoglobin.*—Because of an in-
terruption which will prevent resumption of our experiments
for several months, we are publishing our other data before the
direct determination of the value of β_R, from the slope of the $\dfrac{dB}{dpH}$
line in reduced solution of crystallized hemoglobin, has been com-
pleted. In Table VIII of the accompanying paper (11), however,

we have estimated, from accurately determined differences between the buffer value of blood in the oxygenated and reduced conditions. respectively, that if β_O is 2.64 as determined in this paper, β_R is 2.45.

TABLE IV.

Crystalline Horse Hemoglobin No. 14.

Total hemoglobin content of solution.....7.25   mM. Colorimetric.
                            7.54   "   Kjeldahl.
                            7.40   "   Mean.

Oxygen capacity of solution..............6.37   "

$$\frac{\text{Oxygen capacity}}{\text{Total hemoglobin content}} \dots\dots\dots\dots\dots 0.860$$

Conductivity of saturated HbO_2 solution in H_2O..3.9×10^{-5} mhos.
Concentration of NaOH added to solution................30 mM.

Equilibration Data.

No.	p_{O_2}	Total $[O_2]$.	$[HbO_2]$	P_{CO_2}	Total $[CO_2]$.	$[BHCO_3]$	$[BHb] + [BHbO_2]$	pH	$[BHb] + [BHbO_2]$ at pH 7.42.
	mm.	*mM.*	*mM.*	*mm.*	*mM.*	*mM.*	*mM.*		*mM.*
1	137.6			30.3	17.66	16.72	13.28	7.428	
2	134.6	6.26	6.08	31.9	17.95	16.96	13.04	7.413	13.18
3	143.9	6.22	6.03	60.7	22.58	20.69	9.31	7.220	
4	34.4	5.68	5.63	33.5	18.44	17.40	12.60	7.403	12.90
5	10.6	2.77	2.76	36.9	20.47	19.32	10.68	7.406	10.92
6	2.8	0.60	0.60	37.0	21.67	20.52	9.48	7.431	9.21
					21.78	20.63	9.37	7.433	
7	22.1	4.87	4.85	33.2	18.70	17.67	12.33	7.433	11.95
					18.87	17.84	12.16	7.437	

The Base Bound by Oxyhemoglobin and Reduced Hemoglobin at pH 7.4.

Oxyhemoglobin.—If we take the intersections with the pH 7.4 line, of the lines indicating base bound as $BHbO_2$ and passing through the completely oxygenated points on Figs. 1, 2a, 3a, and 4a, we find that the amounts of alkali bound have the values indicated in Table VI, the average being 2.09 atoms of Na bound by 1 molecule of oxyhemoglobin.

To the data taken from previous tables we have added those from two experiments with Preparations 6 and 10, which have not been reported in detail, because determinations of points of partial oxygenation were lacking. The percentages of hemo-

Fig. 4 a. From the line through the oxygenated points 1, 2, and 3, $\dfrac{dB}{dpH}$ is calculated as 19.3, and β_0 as 2.63.

Intersections of lines through the other points with the pH 7.42 line indicate effects of varying degrees of reduction in lowering the amount of base bound by the hemoglobin.

The numbers near the points indicate the data in Table IV from which they are taken. In the last column of the table the amounts of base indicated by the intersections on the pH 7.42 line are given.

globin capable of binding oxygen in these preparations were 97 and 92, respectively.

It is probable that in Experiment 4 less alkali was added than was estimated. This error would not affect the determination of pH or β_0 or of the $\dfrac{dB}{d[HbO_2]}$ line, since these are all based on

differences in [BHCO$_3$] and not on absolute values. It would invalidate calculations of the [BHbO$_2$] value, however, and we have left this determination out in averaging the results.

Reduced Hemoglobin.—In Figs. 2b, 3b, and 4b the height of the B, [HbO$_2$] lines at the abscissa representing complete reduction ([HbO$_2$] = 0) indicates the amounts of base bound by the

FIG. 4 b. Amounts of base bound by hemoglobin at pH 7.42 compared with amounts of oxygen bound. Data from Table IV and Fig. 4 a.

From the slope of the line, $\frac{dB}{d[HbO_2]}$ is calculated as 0.73.

completely reduced hemoglobin. From the values thus found, in Figs. 2b, 3b, 4b, and similar figures representing data obtained from Preparations 6 and 10, the results in Table VII as "Na bound per mol Hb" are calculated. From these figures the data in the last column of Table VII, at pH 7.4, for comparison with the results in Table VI, are calculated.

TABLE V.

Summary of Experiments to Determine pK' of Hemoglobin Solutions.

Technique:

Double tonometer "First equilibration method." Double saturation.

Saturation at 38° with $H_2 + CO_2$.

Gas phase analyzed in Haldane apparatus.

Liquid phase analyzed for CO_2.

pH of liquid phase determined electrometrically at 38° at CO_2 tension of equilibration.

Solution:

Twice recrystallized HbO_2 plus NaOH to 30 mM.

Date.	Hb preparation.	Total B concentration.	Total Hb concentration.	P_{CO_2}	Total [CO₂].	[BHCO₃]	$R = \frac{[BHCO_3]}{[H_2CO_3]}$	log R	Electrometric pH *	pK'
1922		*mM*	*mM.*	*mM.*	*mM.*	*mM.*				
May 5	Horse 7	30	6.71	52.2	21.44	19.81	12.19	1.086	7.26	6.17
" 5	" 7	30	6.71	84.4	25.55	22.92	8.74	0.941	7.08	6.14
June 1	" 11	30	7.13	48.9	22.05	20.53	13.50	1.130	7.31	6.18
" 16	Dog 16	30	6.27	38.3	25.23	24.05	20.36	1.309	7.50	6.19
" 16	" 16	30	6.27	38.5	25.52	24.33	20.48	1.311	7.50	6.19
Average..										6.18

* Electrometric pH determinations are based on 0.1 N HCl assuming 1.090 as its pH at 38°.

TABLE VI.

Base Bound by Oxyhemoglobin at pH 7.4.

Experiment No	Preparation No.	Total hemoglobin content, C.	Na bound* as Na HbO₂ at pH 7.4	Na bound per mol hemoglobin at pH $7.4 = \frac{[Na\,Hb\,O_2]}{C}$	a calculated $= 7.4 - \frac{[Na\,HbO_2}{2.64\,C}$
		mM.	*mM.*	*mol*	
1	5	7.97	17.23	2.16	6.58
2	7	6.92	14.80	2.14	6.59
3	11	7.64	15.53	2.06	6.62
4	14	7.40	12.80	1.74	6.74
—	6	7.58	15.60	2.06	6.62
—	10	8.87	18.20	2.05	6.62
Average†...................................				2.09	6.61

*. By interpolation on line connecting oxygenated points of Figs. 1, 2a, 3a, and 4a.

† Preparation No. 14 left out of average.

The Difference Between the Base Bound by Reduced and by Oxygenated Hemoglobin.

The difference between the average equivalents of Na bound per molecule of oxygenated and reduced hemoglobin respectively is, from Tables VI and VII, $2.09 - 1.51 = 0.58$. However, the hemoglobin preparations used were on the average capable of taking up and giving off only 0.85 molecule of O_2. So that the real average change of Na per mol change in combined O_2 was approximately $\dfrac{0.58}{0.85} = 0.68$ equivalents of Na.

TABLE VII.

Base Bound by Reduced Hemoglobin.

Experiment No.	Preparation No.	Total hemoglobin content, C.	pH	Na bound as [Na Hb] at pH indicated.	Na bound per mol Hb $= \dfrac{[\text{Na Hb}]}{C}$	b calculated as $= \text{pH} - \dfrac{[\text{Na Hb}]}{2.45\,C}$	Na estimated[*] bound at pH 7.40 per mol Hb.
		m.m.		m.m.	mol		mol
2	7	6.92	7.30	8.86	1.28	6.78	1.52
3	11	7.13	7.31	9.05	1.27	6.78	1.49
4	14	7.40	7.42	8.75	1.18	6.94	1.13
—	6	7.58	7.35	10.45	1.38	6.82	1.50
—	10	8.87	7.30	11.60	1.31	6.77	1.55
Average[†]						6.79	1.51

[*] Estimated by adding 2.45 $(7.4 - \text{pH})$ to Na bound at observed pH.
[†] Preparation No. 14 left out of average.

The values of the ratio $\dfrac{dB}{d[\text{HbO}_2]}$ determined in Experiments 2, 3, and 4, were 0.74, 0.69, and 0.73, respectively. It appears that we may state that the ratio is approximately 0.7 for horse hemoglobin. How much error in it we would allow for is not yet certain. It may be as great as $\pm$ 0.1, but is probably not over $\pm$ 0.05. We hope to establish it more accurately later.

The presence of inactive hemoglobin in our preparations widens the margin of error that must be allowed for in the determination of the absolute amounts of base bound per molecule of oxygenated and reduced hemoglobin. If the inactive hemoglobin has the base-binding power of oxyhemoglobin, then the average figure

2.09 given in Table VI is correct, but this figure for reduced hemoglobin should be lowered by about 0.1. If, however, the inactive hemoglobin has the base-binding power of reduced hemoglobin, the completely oxygenated preparation must bind about 2.2 equivalents of base. The preparation of entirely inactive hemoglobin and the determinations of its base-binding powers are required to settle the nature of the correction it necessitates. Until this has been accomplished we must make allowance for the margin of error that may be occasioned in the figures for either oxygenated or reduced hemoglobin. Such allowance extends the range of results for reduced hemoglobin from 1.55–1.49 to 1.55–1.39 (or 1.47 ± 0.08 equivalents of base per molecule of hemoglobin). It extends the range of results for oxyhemoglobin from 2.05–2.16 to 2.05–2.26, or 2.15 ± 0.10 equivalents.

The Base Bound by Oxyhemoglobin at Varying pH.

In order to calculate the base bound by oxyhemoglobin at varying pH ranges we utilize the fact that the buffer ratio $\frac{dB}{dpH}$ is constant over the physiological range, and has a value of 2.64. This means that as oxyhemoglobin combines with more alkali the pH increases at a constant proportion, the change being at the rate of 1 pH per 2.64 molecules of monovalent base (over the limited range, pH 7.2 to 7.5 at least). Expressed in the usual form of a straight line equation, we have

$$B = [BHbO_2] = 2.64\,[HbO_2]\,(pH - a) \tag{1}$$

where a is the pH at which the base bound would become zero, if the slope $\frac{dB}{dpH}$ continued unaltered to that point. If the $\frac{dB}{dpH}$ value remained absolutely constant up to that point, the latter would be the isoelectric point of oxyhemoglobin. As a matter of fact a is quite close to this point, but we are not at present certain how close. Consequently, it appears better to designate the extrapolated point merely as a, rather than by a symbol which might indicate a relation to the exact isoelectric point. The value of a, found by solving the above equation, is

$$a = pH - \frac{[BHbO_2]}{2.64\,C} \tag{2}$$

From the data of Table VI we calculate in the last column of the table that the value of a lies between 6.58 and 6.62, the average being 6.61. However, the values in Table VI are uncorrected for the inactive hemoglobin. The average corrected value at pH 7.4 for $\dfrac{B}{C}$, $viz.$ 2.15, gives $a = 6.585$.

Similarly we could estimate from points of known pH and B the value of b in the similar equation for reduced hemoglobin,

$$[\text{BHb}] = \beta_R \, [\text{Hb}] \, (\text{pH} - b) \tag{3}$$

From the data in Paper IV of this series (11), β_R is estimated as 2.45. From the average value of $\dfrac{[\text{BHb}]}{C}$, corrected for inactive hemoglobin, $viz.$ 1.47 at pH 7.4, we obtain by calculation analogous to that of Equation 2, a value of 6.80 for b. This we use instead of the uncorrected average value of 6.79 given in Table VII.

$$[\text{BHb}] = 2.45 \, [\text{Hb}] \, (\text{pH} - 6.80) \qquad \cdot \qquad \tag{4}$$

Finally, by combining Equations 1 and 4 we obtain an expression relating the total base bound by hemoglobin to the pH and the per cent of oxygen saturation. Expressing the total base $[\text{BHbO}_2] + [\text{BHb}]$ as B, we have, by addition of Equations 1 and 4, Equation 5, which indicates the relationships of all three variables.

$$B = 2.64 \, [\text{HbO}_2] \, (\text{pH} - 6.585) + 2.45 \, [\text{Hb}] \, (\text{pH} - 6.80) \tag{5}$$

By substituting $C - [\text{HbO}_2]$ for $[\text{Hb}]$ we may solve Equation 5 in terms of $[\text{HbO}_2]$ as

$$B = 2.45 \, C \, (\text{pH} - 6.80) + (0.19 \, \text{pH} - 0.72) \, [\text{HbO}_2] \tag{6}$$

Table VIII contains a comparison of observed and calculated values for $[\text{BHb}] + [\text{BHbO}_2]$, the "observed" being taken from the tables, the "calculated" being calculated by Equation 6. In making the calculations the inactive hemoglobin was calculated as HbO_2. How much the observed deviation of about 1.0 mm. in No. 3 is due to error from this assumption is uncertain. The deviation in No. 4 is due to the fact, stated before, that almost certainly 3 mm. more alkali were added in the experiment than was intended.

In Equation 6 the term 2.45 C (pH − 6.80) represents the base that would be bound if all the hemoglobin were reduced, while the term (0.19 pH − 0.72) [HbO$_2$] represents the additional amount of base combined at the observed pH as the result of oxygenation of part of the hemoglobin, the coefficient (0.19 pH − 0.72) representing $\dfrac{dB}{d[HbO_2]}$.[1] This amount in equivalents of base added per mol of oxygen is, according to the results in the present paper, about 0.7 at pH 7.4. Calculated as 0.19 pH − 0.7, it is 0.67 at pH 7.30, 0.69 at pH 7.40, 0.71 at pH 7.50.

TABLE VIII.

Comparison of Observed and Calculated [BHbO$_2$] + [BHb] Values.

Experiment No.	Table I.		Table II.		Table III.		Table IV.	
	Calcu-lated.	Ob-served.	Calcu-lated.	Ob-served.	Calcu-lated.	Ob-served.	Calcu-lated.	Ob-served.
1	20.1	20.0	14.9	14.7	15.9	15.5	16.3	13.3
2	19.2	19.4	11.2	11.1	12.4	11.6	15.9	13.0
3	18.2	18.4	9.0	8.6	10.8	9.8	10.9	9.3
4	15.9	16.4			10.3	9.5	15.4	12.6
5	15.8	16.5	10.8	10.9	12.8	12.1	13.6	10.7
6	15.6	15.8	11.7	11.2	12.8	12.2	11.5	9.4
7	14.5	14.7	11.3	11.8	13.0	12.5	14.6	12.2
8	14.4	14.4	10.6	10.3	13.1	12.5		
9	13.7	13.9	10.8	10.6	10.8	10.0		
10	10.1	11.0			11.1	10.3		

Equation 5 and its derivatives are, of course, purely empirical, and hold only for the range over which β_O and β_R are constant. They afford, however, a convenient way of stating the observed relationships over at least the greater part of the physiological range of blood pH.

[1] As a matter of fact, Equation 6 was the first expression actually developed for empirically expressing the relationships of the three variables pH, B, and oxygen. It was developed by Hastings, who noted in charting Experiments 4, 5, and 6 in the next paper (11) that $\dfrac{dB}{d[HbO_2]}$ increases with pH according to a linear function of the latter, and might therefore be expressed in the form m pH $+ n$.

An equation attempting to express the base bound at varying pH in terms of the mass law would have the form

$$B = C \left(\frac{[HbO_2]}{C} \frac{K_O}{K_O + [H^+]} + \frac{[Hb]}{C} \frac{K_R}{K_R + [H^+]} + \frac{K_3}{K_3 + [H^+]} + \frac{K_4}{K_4 + [H^+]} + \cdots \right)$$

with at least six terms, since as shown above, there are at least five and probably more acid groups binding more or less base at normal blood pH in both oxygenated and reduced hemoglobin.

As L. J. Henderson has shown (6), assuming that changes in one acid group in hemoglobin are responsible for the difference in alkali-binding power between oxygenated and reduced hemoglobin, the group affected would have a relatively low dissociation constant, K_R, in reduced hemoglobin and a relatively high one, K_O, in oxygenated hemoglobin; and the value of K_O and K_R could be calculated from the difference in base bound by oxygenated and reduced hemoglobin at two different pH points. All our results are consistent with Henderson's assumption; and the values of K_O and K_R could be calculated from the difference in base bound by oxygenated and reduced hemoglobin at pH 7.2 and 7.4 estimated from our average data as expressed by Equation 5 or 6. The change in $\frac{dB}{d[HbO_2]}$ with changing pH on which one must base calculations of K_O and K_R is slight, however, and it seems preferable to delay such calculation until experiments with hemoglobin solutions similar to Experiments 4, 5, and 6 with blood in the accompanying paper (11) are available, and a wider range of pH is covered so that the validity of the assumption can be tested by the constancy of results obtained with varying pairs of pH points.

The technique used in this paper may be of value in characterizing other proteins by determining the height and slope (buffer value) of their B, pH curves on the alkaline side of their isoelectric points. We have estimated above that the pH values determined are reproducible with a maximum error of 0.007 pH; the usual error need not exceed half as much. Such constancy appreciably exceeds that attainable by the electrometric method.

SUMMARY.

1. The alkali (Na) bound at pH 7.4 per gram molecule of recrystallized horse oxyhemoglobin has been determined to be 2.15 $\pm$ 0.10 equivalents, that per gram molecule of reduced hemoglobin to be 1.47 $\pm$ 0.08. The errors of $\pm$ 0.10 and $\pm$ 0.08 allowed for are about half due to the combined analytical errors connected with the technique, and half to the presence of 7 to 20 per cent of hemoglobin in a form incapable of binding labile oxygen in the preparations used. (In these calculations a gram molecule of hemoglobin has been taken as the amount combining with a gram molecule of oxygen.)

2. At pH 7.4, the change of 1 mol of reduced hemoglobin to oxyhemoglobin enables the hemoglobin to combine with 0.68 $\pm$ 0.10 equivalent of additional alkali.

3. For points intermediate between complete oxygenation and complete reduction, increase in base bound at constant pH is in simple direct proportion to increase in oxygen content. The $\dfrac{dB}{d[HbO_2]}$ ratio at a given pH is constant for all degrees of oxygenation. This fact accords with Henderson's theory (6).

4. The variation in base bound by oxyhemoglobin at varying pH is indicated by the molecular buffer value of $\beta_O = 2.64$. For each 0.1 pH increase, each molecule of oxyhemoglobin takes up 0.264 additional equivalents of base.

For reduced hemoglobin the molecular buffer value β_R is 2.45.

These buffer values are practically constant over the pH range 7.2 to 7.5.

5. The relationships summarized above are condensed into the empirical equation, valid over the pH range 7.2 to 7.5, and approximately so to pH 7.0 to 7.6:

$$B = 2.64\,[HbO_2]\,(pH - 6.585) + 2.45\,[Hb]\,(pH - 6.80)$$

The constants, 2.45 and 6.80, of the last term have been determined indirectly, and may be subject to slight revision when direct determinations are available.

6. The molecular buffer values, $\beta_O = 2.64$ and $\beta_R = 2.45$, indicate that at pH 7.2 to 7.5 at least five monovalent acid groups in the molecule share in the alkali combined with hemoglobin.

7. The constants above reported are for horse hemoglobin only. Preliminary experiments indicate that for dog hemoglobin the figures are different.

BIBLIOGRAPHY.

1. Austin, J. H., Cullen, G. E., Hastings, A. B., McLean, F. C., Peters, J. P., and Van Slyke, D. D., *J. Biol. Chem.*, 1922, liv, 121.
2. Van Slyke, D. D., *J. Biol. Chem.*, 1922, lii, 525.
3. Van Slyke, D. D., *Physiol. Rev.*, 1921, i, 141.
4. Christiansen, J., Douglas, C. G., and Haldane, J. S., *J. Physiol.*, 1914, xlviii, 244.
5. Joffe, J., and Poulton, E. P., *J. Physiol.*, 1920, liv, 129.
6. Henderson, L. J., *J. Biol. Chem.*, 1921, xlvi, 411.
7. Peters, J. P., Barr, D. P., and Rule, F. D., *J. Biol. Chem.*, 1920–21, xlv, 489.
8. Heidelberger, M., *J. Biol. Chem.*, 1922, liii, 31.
9. Van Slyke, D. D., *Proc. Nat. Acad. Sc.*, 1921, vii, 229.
10. Bohr, C., *Skand. Arch. Physiol.*, 1905, xvii, 104.
11. Van Slyke, D. D., Hastings, A. B., and Neill, J. M., *J. Biol. Chem.*, 1922, liv, 507.

STUDIES OF GAS AND ELECTROLYTE EQUILIBRIA IN THE BLOOD.

IV. THE EFFECT OF OXYGENATION AND REDUCTION ON THE BICARBONATE CONTENT AND BUFFER VALUE OF BLOOD.

By DONALD D. VAN SLYKE, A. BAIRD HASTINGS, AND
JAMES M. NEILL.

(From the Hospital of The Rockefeller Institute for Medical Research.)

The experiments here presented were carried out in order—

1. To determine the buffer value of oxygenated and reduced blood, and the proportion of it for which hemoglobin is responsible.

2. To ascertain quantitatively the effect of change in combined oxygen at constant pH on the blood bicarbonate.

3. To ascertain the effect of changing pH on the $\dfrac{d[BHCO_3]}{d[HbO_2]}$ ratio.[1]

We have used the same experimental methods employed with hemoglobin solution (1). The blood was saturated at 38° under known tensions of CO_2 and O_2, and the changes in base distribution were estimated from changes in the bicarbonate.

In the experiments with hemoglobin solutions (1) all changes in base present as $BHCO_3$ could be interpreted as changes in the opposite direction in base bound by hemoglobin, since carbonic acid and hemoglobin were the only acids present. Blood, however, also contains other buffers, chiefly in the form of plasma proteins and cell phosphate, which also give off some bases to form bicarbonate when pH is lowered by increased CO_2 tension. Consequently, variations in $[BHCO_3]$ accompanying changing pH due to changing CO_2 tension cannot be interpreted on blood as quantitative measures of opposite changes in $[BHb] + [BHbO_2]$. However, we may estimate the buffer value of oxyhemoglobin

---

[1] The symbols used in the preceding paper (1) are employed.

from its concentration and its molecular buffer values determined
in the preceding paper (1), and by comparing the values thus
estimated with the observed total buffer values we may ascer-
tain the relative part played by oxyhemoglobin in the total buffer
effect of the blood. The estimation from buffer constants ob-
tained on hemoglobin solutions, of the buffer effect of hemoglobin
in the blood involves the assumption that the confinement of
hemoglobin within the red cells does not alter its buffer effect on
the system. The approximate correctness of this assumption is
shown by the results of Joffe and Poulton (2). Its absolute
accuracy must be based on parallel experiments with laked and
intact cells, which the temporary interruption of our work has
forced us to leave to the future. The calculation of the relative
part played by hemoglobin in the buffer effect of blood in Table
VII is therefore presented with the reservation that slight cor-
rections may be required because of the difference in pH between
plasma and cell contents. Because of the relative constancy of
the buffer value of hemoglobin over the pH range that the cell
contents presumably cover, however, it does not appear that
the correction can be considerable.

If we keep the pH of blood constant, however, by properly
regulated CO_2 tension, and change only the degree of oxygenation,
we appear justified in assuming that any changes in $[BHCO_3]$
that occur correspond quantitatively to changes in the opposite
direction in base bound by hemoglobin. For hemoglobin is the
only substance in the blood that combines with the oxygen, and
can therefore conceivably undergo change in its chemical properties
from oxygenation and reduction of the blood. Expressing these
increases by the symbol Δ, with changing oxygen content at
constant pH, $- \Delta[BHCO_3] = \Delta([BHb] + [BHbO_2])$.

EXPERIMENTAL.

The blood in volume from 250 to 400 cc. was drawn from the
horse the night before each experiment. It was either oxalated
or defibrinated, as described before (3), was chilled to nearly
0°C., and was kept at that temperature until samples were re-
moved for saturation the next morning. For the oxalation 0.2
per cent potassium oxalate plus 0.1 per cent of sodium fluoride
was used (4).

The technique of saturation and analysis was identical with that used in the preceding paper (1) for hemoglobin solutions. For the blood CO_2 determinations 3 cc. samples were used.

The calculations of pH values were also performed as above (1), except that slightly different constants were required. For calculation of the pH values the value 6.15 for pK' was employed, instead of 6.18 as with hemoglobin solutions. The data on which the value 6.15 is based will be published later. For α_{co_2}, Bohr's value of 0.511 for whole blood was employed.

Our experiments are divided into two groups.

In Experiments 1, 2, and 3 we have followed the procedure outlined for Experiments 1, 3, and 4 of the preceding paper (I). The slope of the $BHCO_3$, pH line has been determined on oxygenated blood, and used to extrapolate to a given pH the $[BHCO_3]$ values found in bloods in varying stages of oxygenation, and at pH's as near to the given pH as could be arranged by regulation of the CO_2 and O_2 tensions. By these experiments we obtained data on the buffer value of oxygenated blood, on the effect of oxygenation and reduction on $[BHCO_3]$ at constant pH, and on the proportionality between this effect and the degree of oxygenation. The results are given in Tables I, II, and III, and in Figs. 1a, 1b, 2a, 2b, 3a, and 3b.

In order to obtain data also on the buffer value of reduced blood, and on the total effect of reduction and oxygenation at varying pH, we have carried out Experiments 4, 5, and 6. In each of these a number of $[BHCO_3]$ values were obtained with blood approximately completely oxygenated, $p_{O_2} = 140$ mm. at varying pH, and a number of other $[BHCO_3]$ values were obtained on blood as near as possible to complete reduction. The $[BHCO_3]$ points for both oxygenated and reduced bloods when plotted against pH made absolutely straight lines within the pH range of the experiments, 7.1 to 7.5.

The results of Experiments 4, 5, and 6 are given in Tables IV a, IVb, Va, Vb, VIa, and VIb, and in Figs. 4, 5, and 6. In Tables IVb, Vb, and VIb, the values for $\dfrac{d[BHCO_3]}{d[HbO_2]}$ may be taken, for reasons discussed above, to represent negative values of $\dfrac{dB}{d[HbO_2]}$ in the sense used in the preceding paper, B indicating the total base bound by hemoglobin as $[BHb] + [BHO_2]$ (1).

EXPLANATION OF TABLES AND FIGURES.

In all except Tables IV*b*, V*b*, and VI*b* the symbols used are the same as in the tables of the preceding paper. In Tables IV*b*, V*b*, and VI*b*, we have used $[BHCO_3]_R$ for the $[BHCO_3]$ of the reduced blood; $[BHCO_3]_O$ for the $[BHCO_3]$ of the oxygenated blood; $\Delta[BHCO_3] = [BHCO_3]_R - [BHCO_3]_O$. The ratio $\dfrac{\Delta[BHCO_3]}{\Delta[HbO_2]}$ is always negative since at constant pH, oxygen and bicarbonate contents move in opposite directions.

The charts are designed like those in the preceding paper, except that in the present one the $[BHCO_3]$ values are plotted as ordinates, instead of the $[BHb] + [BHbO_2]$ values. Consequently, since the $[BHCO_3]$ varies in the opposite direction from the $[BHb] + [BHbO_2]$ in the present charts the lines slope in the opposite direction from those in the preceding paper, and the oxygenated line is at the bottom of a series instead of at the top.

TABLE I.

Horse Blood No. 1.

Anticoagulant . . . . .Oxalate + NaF.
Total oxygen capacity. . . . . .7.30 mM.

No.	Po_2	Total $[O_2]$.	$[HbO_2]$	Pco_2	Total $[CO_2]$.	$[BHCO_3]$	pH	$[BHCO_3]$ at pH 7 30.
	mm.	*mM.*	*mM.*	*mm.*	*mM.*	*mM.*		*mM.*
1	(140)	7.39	7.21	33 2	16.05	15.05	7.329	15.75
2	(140)	7.36	7.18	47.1	18.64	17.23	7.236	15.75
3	(140)	7.38	7.20	61.7	20.90	19.05	7.162	15.75
4	49.30	5.75	5.68	44 4	18.55	17.22	7.265	16.40
5	34.50	4.49	4.45	44.4	19.05	17.72	7.274	17.10
6	32.70	4 29	4.25	44.2	19.15	17.82	7.278	17.28
7	21.60	2.26	2.24	46.6	20.35	18.95	7.282	18.55
8	2.9	0 32	0 32	51 4	21.76	20.22	7.268	19.47
9	142.0	7.38	7.20	40.6	17.52	16.30	7.276	15.75

DISCUSSION OF RESULTS.

The Buffer Value of Oxygenated and Reduced Blood and the Proportion of It for Which Hemoglobin Is Responsible.

The buffer values for oxygenated blood, in mM. terms, are given in Table VII for the five bloods on which they were determined.

FIG. 1a. From the line through the oxygenated points 1, 2, 3, and 9, $\frac{d[BHCO_3]}{dpH}$ is calculated as -23.7.

Intersections of lines through the other points with the pH 7.3 line indicate effects of varying degrees of reduction in lowering the amount of base bound by the hemoglobin.

The numbers near the points indicate the data in Table I from which they are taken. In the last column of the table the amounts of base indicated by the intersections on the pH line are given.

FIG. 1b. Amount of base displaced from $BHCO_3$ at pH 7.3 compared with amounts of oxygen bound. Data from Table I and Fig. 1a.

From the slope of the line, $\frac{d[BHCO_3]}{d[HbO_2]}$ is calculated as -0.554.

511

(On No. 3 the buffer slope was not determined, but was estimated for purposes of extrapolation from the hemoglobin content.) The value of the buffers other than $BHCO_3$ was estimated as $\dfrac{dB}{dpH} = -\dfrac{d[BHCO_3]}{dpH}$ since increase in base bound as $BHCO_3$ is equal to loss of base by other buffers. The other calculations in the table are self-explanatory. It appears that from 73 to 79 per cent of the total buffer effect of the oxygenated horse blood was due to oxyhemoglobin. The average buffer effect of

TABLE II

Horse Blood No. 2.

Anticoagulant.................................Oxalate and NaF.
Total oxygen capacity.................................6.80 mM.

No.	Po_2	Total $[O_2]$	$[HbO_2]$	Pco_2	Total $[CO_2]$	$[BHCO_3]$	pH	$[BHCO_3]$ at pH 7 275.
	mm	*mM.*	*mM*	*mm.*	*mM.*	*mM.*		*mM*
1	143 3	6 93	6 75	33 0	16.79	15.80	7 353	17.35
2	142.8	6.95	6.77	42 3	18.56	17.29	7.274	17.35
3	(143.0)	6 86	6 68	62.2	21.63	19 57	7.170	17.35
4	44 2	5.26	5 20	44.7	19.47	18.13	7.271	18.6,
5	31 5	3 85	3 81	44.7	20.06	18.72	7.298	19.26
6	27 5	3 03	2.99	45.6	20.40	19.03	7.293	19.42
7	21.0	1.90	1 87	49.5	21.45	19.96	7.278	20.04
8	1.5	0 14	0.14	48.7	22.13	20.67	7 291	20.98
9	141 4			42.4	18.51	17.24	7.282	17.35

buffers other than bicarbonate, in mM. terms, was $\dfrac{dB}{dpH} = 22.6$, of which 19.0 were due to hemoglobin, leaving 3.6 to buffers other than hemoglobin and bicarbonate. The bicarbonate contributed an average $\dfrac{dB}{dpH}$ value of 1.8, making the total 24.4.

In percentages of the total buffer effect, the average values for all five bloods are 78 per cent for hemoglobin, 7.4 per cent for bicarbonate, and 14.6 for all other buffers.

The results obtained with reduced blood in Experiments 4, 5, and 6 are given in Table VIII. The effects of buffers other than hemoglobin and $BHCO_3$, are estimated in Column d of Table VIII from the oxygenated samples by subtracting the

FIG. 2a. From the line through the oxygenated points 1, 2, 3, and 9, $\frac{d[BHCO_3]}{dpH}$ is calculated as -20.6.

Intersections of lines through the other points with the pH 7.275 line indicate effects of varying degrees of reduction in lowering the amount of base bound by the hemoglobin.

The numbers near the points indicate the data in Table II from which they are taken. In the last column of the table the amounts of base indicated by the intersections on the pH line are given.

FIG. 2b. Amounts of base displaced from BHCO₃ at pH 7.275 compared with amounts of oxygen bound. Data from Table II and Fig. 2a.

From the slope of the line, $\frac{d[BHCO_3]}{d[HbO_2]}$ is calculated as -0.548.

calculated buffer effect of the oxyhemoglobin from the total. In the reduced bloods it is assumed that the effects of the buffers other than $BHCO_3$ and hemoglobin are the same as in oxygenated. The buffer value of the reduced hemoglobin is accordingly estimated by subtracting the values in Column d from those in Column e. From the $\dfrac{dB}{dpH}$ values found, the molecular buffer value is calculated in Column g as $\beta_R = \dfrac{dB}{CdpH}$.

From comparison in Tables VII and VIII of the average values for those experiments in which both oxygenated and re-

TABLE III.

Horse Blood No. 3.

Anticoagulant....................................Defibrinated.
Total oxygen capacity..............................6 68 mM.

No.	P_{O_2}	Total $[O_2]$.	$[HbO_2]$	P_{CO_2}	Total $[CO_2]$	$[BHCO_3]$	pH	$[BHCO_3]$ at pH 7 24.
	mm.	*m*M.	*m*M.	*mm.*	*m*M.	*m*M.		*m*M.
1	143.5	6.75	6.56	41.9	16.65	15.39	7.238	15.38
2	37.6	4.30	4.25	46.0	18.14	16.76	7.235	16.63
3	19.9	1.90	1.87	47.3	19.08	17.66	7.245	17.74
4	4.0	0.22	0.22	48 7	19.91	18.45	7.251	18.67
5	135.0	6.69	6.49	43.4	16.80	15.50	7.226	15 25
6	29.70	3.21	3.17	45.0	18.13	16.78	7.244	16 85
7	20.80	2.00	1.97	46.2	18.85	17.46	7.250	17 66
8	3.5	0.19	0.19	48.7	19.93	18.47	7.252	18 73

duced bloods were examined, the shift of buffer values resulting from reduction of oxygenated blood at constant pH becomes apparent. The hemoglobin decreases in both percentage and absolute buffer effect, because of the chemical change it undergoes; the bicarbonate increases in both percentage and total buffer effect, because of the increase in $[BHCO_3]$, and its gain nearly balances the loss of the hemoglobin.

The Effect of Change in Combined Oxygen at Constant pH on the Blood Bicarbonate.

The values for the $\dfrac{d[BHCO_3]}{d[HbO_2]}$ ratios obtained at 7.30, or the

FIG. 3a. The slope of the lines is calculated as $\frac{C\beta_0}{0.85}$, from the hemoglobin content.

Intersections of lines through the other points with the pH 7.24 line indicate effects of varying degrees of reduction in lowering the amount of base bound by the hemoglobin.

The numbers near the points indicate the data in Table III from which they are taken. In the last column of the Table the amounts of base indicated by the intersections on the pH 7.24 line are given.

FIG. 3b. Amounts of base displaced from $BHCO_3$ at pH 7.24 compared with amounts of oxygen bound. Data from Table III and Fig. 3a.

From the slope of the line, $\frac{d[BHCO_3]}{d[HbO_2]}$ is calculated as -0.538.

TABLE IV*a*.

Horse Blood No. 4.

Anticoagulant.....................................Oxalate + NaF.
Total oxygen capacity..............................7.16 mM.
Technique.....................................Double tonometer.
                                         Double equilibration.

No.	P_{O_2}	Total [O_2].	[HbO$_2$]	P_{CO_2}	Total [CO$_2$].	[BHCO$_3$]	pH
	mm.	*m*M.	*m*M.	*mm.*	*m*M.	*m*M.	
1	136.9	7.30	7.12	54.7	19.55	17.91	7.188
2	136.2	7.27	7.09	53.8	19.35	17.74	7.191
3	128.2			31.0	15.25	14.32	7.338
4a	130.3	7.32	7.15	24.2	13.57	12.84	7.398
4b	130.6	7.31	7.14	24.2	13.50	12.77	7.395
Average.............			7.13				
5	2.6	0.22	0.22	63.0	23.27	21.38	7.203
6	3.3	0.25	0.25	46.0	20.66	19.28	7.295
7	3.5	0.31	0.31	34.2	18.44	17.41	7.379
8	3.6	0.33	0.33	25.8	16.43	15.66	7.456
Average.............			0.28				

TABLE IV*b*

Horse Blood No. 4. Data from Fig. 4.

pH	[BHCO$_3$]$_R$	[BHCO$_3$]$_O$	Δ[BHCO$_3$]	$-\dfrac{\Delta[BHCO_3]^*}{\Delta[HbO_2]}$
	*m*M.	*m*M.	*m*M.	
7.1	23.70	20.00	3.70	0.540
7.2	21.46	17.59	3.87	0.565
7.3	19.20	15.16	4.04	0.589
7.4	16.96	12.75	4.21	0.615
7.5	14.70	10.32	4.38	0.639

*Δ[HbO$_2$] = 0.28 − 7.13 = − 6.85

nearest point to 7.30, covered by observation, are given in Table IX.

The assumption appears justified, as stated above, that, at constant pH, changes in oxygenation affect the amounts of base bound only by carbonic acid and by hemoglobin, whatever base is detached from combination with hemoglobin being represented by increase in [BHCO$_3$], and *vice versa*. If, therefore, no factors

in blood alter the pK_R' and pK_O' values of the acid group or groups of which the base-binding power is affected by oxygen changes, we should expect at a given pH the $\dfrac{dB}{d[HbO_2]}$ ratio determined in sodium hemoglobinate solutions to have the same value as the $\dfrac{d[BHCO_3]}{d[HbO_2]}$ ratio determined in blood, except for change in sign.

Fig. 4. From the line through the oxygenated points 1, 2, 3, 4 a, and 4b, $\dfrac{d[BHCO_3]}{dpH}$ for the oxygenated blood is calculated as -24.18. From the line through points 5, 6, 7 and 8, $\dfrac{d[BHCO_3]}{dpH}$ for reduced blood is calculated as -22.40.

The numbers near the points indicate the data in Table IVa from which they are taken.

TABLE Va.

Horse Blood No. 5.

Anticoagulant .... Oxalate + NaF.
Total oxygen capacity .... 6.95 mM.

No.	P_{O_2}*	Total [O_2].	[HbO_2]	P_{CO_2}	Total [CO_2].	[BHCO$_3$]	pH
	mm.	mM.	mM.	mm.	mM.	mM.	
1	(140)	7.13	6.95	52.5	19.28	17.70	7.200
2	(140)			36.3	16.50	15.41	7.301
3	(140)	7.11	6.93	26.5	14.38	13.58	7.382
4	(140)	7.12	6.94	19.9	12.65	12.05	7.455
5	(0)	0.40	0.40	60.8	22.63	20.81	7.207
6	(0)	0.28	0.28	44.5	20.19	18.85	7.299
7	(0)	0.37	0.37	31.3	17.63	16.69	7.400
8	(0)	0.16	0.16	22.5	15.48	14.80	7.491
9	(140)	6.98	6.80	63.1	20.68	18.79	7.148
10	(140)	7.19	7.01	46.2	18.27	16.88	7.236
11	(140)	7.15	6.97	31.3	15.50	14.56	7.341
12	(140)	7.06	6.85	21.5	12.95	12.30	7.431
13	(0)	0.21	0.21	72.1	24.03	21.87	7.155
14	(0)	0.17	0.17	51.9	21.29	19.73	7.253
15	(0)	0.30	0.30	35.9	18.57	17.49	7.361

* P_{O_2} figures in this table represent oxygen tension of gas placed in tonometers for second saturation, not the final tension determined by analysis.

TABLE Vb.

Horse Blood No. 5. Data from Fig. 5.

pH	[BHCO$_3$]$_O$	[BHCO$_3$]$_R$	Δ[BHCO$_3$]	$-\dfrac{\Delta[BHCO_3]^*}{\Delta[HbO_2]}$
	mM.	mM.	mM.	
7.1	19.88	23.00	3.12	0.469
7.2	17.65	20.90	3.25	0.489
7.3	15.42	18.80	3.38	0.508
7.4	13.20	16.70	3.50	0.526
7.5	10.97	14.60	3.63	0.546

* $\Delta[HbO_2] = -6.65$

However, at pH 7.30 the results of sodium hemoglobinate solutions (1) have indicated a value of 0.67 for the $\dfrac{dB}{d[HbO_2]}$ ratio. This is higher than any of the $\dfrac{dB}{d[HbO_2]}$ values obtained in blood,

and it appears probable that the difference is outside the limits of experimental error.

Also it appears that the difference in the value of $\dfrac{dB}{d[HbO_2]}$ as determined at pH 7.3 in Bloods 4 and 5, respectively, is out-

FIG. 5. From the line through the oxygenated points, $\dfrac{d[BHCO_3]}{dpH}$ is calculated as -22.23. From the line through the reduced points $\dfrac{d[BHCO_3]}{dpH}$ for reduced blood is calculated as -21.06.

The numbers near the points indicate the data in Table Va from which they are taken.

side the limits of experimental error. The experiments on these two bloods were performed near the end of our work when the accuracy of our technique was at its best, and a number of points on oxygenated blood and blood at a uniform degree of nearly complete reduction were obtained. Both bloods were oxalated

and otherwise treated in an identical manner. They were also drawn from the same horse at times only 3 days apart. Yet the $\dfrac{dB}{d[HbO_2]}$ ratio in Experiment 4 is 0.589, and in Experiment 5 is 0.508.

TABLE VIa.

Horse Blood No. 6.

Anticoagulant.................................Defibrinated.
Total oxygen capacity.............................7.81 mM.

No.	P_{O_2}*	Total [O_2].	[HbO_2]	P_{CO_2}	Total [CO_2].	[$BHCO_3$]	pH
	mm.	mM.	mM.	mm.	mM.	mM.	
1	(140)	7.86	7 68	52.1	18.32	16.76	7.180
2	(140)	7.81	7.63	37.2	15.66	14.54	7.265
3	(140)	7.97	7.79	25.9	13.21	12.43	7.354
4	(140)	7.91	7.73	18.7	11.23	10.67	7.429
5	(0)	0.78	0.78	59.8	21.57	19.78	7.193
6	(0)	1.04	1.64	43 6	18.94	17.63	7.279
7	(0)	0.98	0.98	31.2	16.52	15.58	7.371
8	(0)	1.01	1.01	22.8	14.38	13.70	7.452

* The P_{O_2} figures in this table represent oxygen tension of gas placed in tonometers for second saturation, not final tension determined by analysis.

TABLE VIb.

Horse Blood No. 6.   Data from Fig. 6.

pH	[$BHCO_3$]$_O$	[$BHCO_3$]$_R$	Δ[$BHCO_3$]	$-\dfrac{\Delta[BHCO_3]^*}{\Delta[HbO_2]}$
	mM.	mM.	mM.	
7.2	16 25	19 54	3.29	0.487
7.3	13 80	17.20	3.40	0.503
7.4	11.37	14.90	3.53	0 522
7.5	8.91	12.59	3.68	0.544

* $\Delta[HbO_2] = -6 76$

There appear to be factors present in the blood which depress the $\dfrac{dB}{d[HbO_2]}$ ratio (B representing base bound by hemoglobin) below the value that it has in solution of sodium hemoglobinate and bicarbonate, and which depress it to a variable extent. One of these factors is probably the difference in pH between cells

and plasma.   The pH values that we have estimated in the blood
are those of the plasma.   The pH values in the cells are probably
lower; and, as will be brought out below, depression of the pH
depresses the $\frac{dB}{d[HbO_2]}$ ratio.

It may be that the difference in concentration of salts in the
blood, as compared with our sodium hemoglobinate solutions,

FIG. 6. From the line through the oxygenated points 1, 2, 3, and 4,
$\frac{d[BHCO_3]}{dpH}$ is calculated as -24.16.   From the line through the reduced
points 5, 6, 7, and 8, $\frac{d[BHCO_3]}{dpH}$ for reduced blood is calculated as -23.23.

The numbers near the points indicate the data in Table VIa from which
they are taken.

also affects somewhat the pK' values of the acid group or groups
in the hemoglobin molecule which are altered by oxygenation.
The question as to whether these two factors are sufficient to
account for the observed variations in the ratio must await the
accumulation of more data.

TABLE VII.

The Relative Part Played by Hemoglobin in the Total Buffer Effect of Oxygenated Blood at pH 7.35.

Blood No.	Condition.	Total buffer value of oxygenated blood exclusive of bicarbonate.* a $\frac{dB}{dpH}$	[BHCO₃] at pH 7.35 b mM.	Buffer value of [BHCO₃] at pH 7.35 = 0.127 [BHCO₃]. c $\frac{dB}{dpH}$	Total buffer value of blood $a + c$. d $\frac{dB}{dpH}$	Hemoglobin content [HbO₂]. e mM.	Estimated oxyhemoglobin buffer effect = 2.61 e. f	Proportion of total buffer effect due to Oxyhemoglobin. per cent	[BHCO₃] per cent	Other buffers. per cent
1	Oxalated.	23.72	15.75	2.00	25.75	7.30	19.27	74.8	7.8	17.4
2	"	20.64	15.80	2.01	22.65	6.80	17.94	79.2	8.9	11.9
4	"	21.18	14.00	1.78	23.96	7.16	18.90	72.8	6.9	20.3
5	"	22.23	11.30	1.83	24.06	6.95	18.35	76.3	7.6	16.1
6	Defibrinated.	24.16	12.60	1.60	25.76	7.72	20.36	79.0	6.2	11.8
Average of Nos. 4, 5, and 6					25.26			76.0	6.9	17.1

* The buffer values are expressed in mM. terms of B.

† pK' for BHCO₃ being 6.15. pH − pK' = 7.35 − 6.15 = 1.20. For pH − pK' = 1.20 Fig. 9 of a former paper on buffers (5) shows 22 per cent of maximum buffer value is exerted. The maximum is 0.575 C, where C is the concentration of the buffer. 0.575 × 0.22 × C = 0.127 C.

TABLE VIII.

Buffer Values in Reduced Blood, and Estimation of β_R in Experiments 4, 5, and 6.

Blood No.	Total hemoglobin content from O₂ capacity.	Values of $\frac{dB(\text{mm.})}{d \, pH}$ exclusive of bicarbonate.				Molecular buffer value of reduced Hb. $\beta_R = \frac{f}{a}$	[BHCO₃] at pH 7.35.	Buffer† value of [BHCO₃] at pH 7.35.	Total buffer value of reduced blood.	Proportion of total effect due to		
		For* [HbO₂] in oxygenated blood. $= a \times \beta_o$	For buffers other than [HbO₂]. $= b - c$	For reduced blood. Observed	Reduced hemoglobin. $= e - d$					Reduced hemoglobin.	[BHCO₃]	Other buffers.
	Observed											
	a	c	d	e	f	a	mm.	$\frac{dB}{dpH}$	$\frac{dB}{dpH}$			
	a	b											
	mm.										percent	percent	percent
4	7.16	18.90	5.28	22.40	17.12	2.40	18.08	2.30	24.70	69.3	9.3	21.1
5	6.95	18.35	3.88	21.06	17.18	2.47	17.75	2.25	23.31	73.7	9.6	16.7
6	7.72	20.36	3.80	23.23	19.43	2.51‡	16.02	2.01	25.27	77.0	8.0	15.0
Average..........						2.45			24.43	73.3	9.0	17.7

*β_o = molecular buffer value of oxyhemoglobin = 2.64 from previous paper.

† See dragger (†) foot-note to Table VII.

‡ This solution was only 88 per cent reduced. It it were completely reduced the value would presumably be 2.49 instead of 2.51. The figure 2.49 is used in calculating the average.

The Effect of Changing pH on the $\dfrac{d[BHCO_3]}{d[HbO_2]}$ Ratio.

From Tables IVb. Vb, and VIb it will be seen that the value of the ratio $-\dfrac{\Delta[BHCO_3]}{\Delta[HbO_2]}$ increases with increasing pH. The rates of increase in the ratio per unit increase in pH are 0.248, 0.192, and 0.190 in Experiments 4, 5, and 6, respectively, or about 0.02 for each 0.1 change in pH over the range pH 7.5 to 7.1.

From the curves of Figs. 4, 5, and 6 it is possible by a method of calculation already outlined by L. J. Henderson (4) to estimate the values K'_R and K'_O of the monovalent acid group in the hemoglobin molecule that has its dissociation constant changed from K_R to K_O when reduced hemoglobin is oxygenated, the

TABLE IX.

Decrease in [BHCO$_3$] per Gram Molecule of Oxygen Combined at Constant pH.

Experiment No.	pH	Treatment of blood.	Mols decrease in [BHCO$_3$] per mol increase in [HbO$_2$].
1	7.30	Oxalate + NaF.	0.554
2	7.275	" + "	0.548
3	7.24	Defibrinated.	0.538
4	7.30	Oxalate + NaF.	0.589
5	7.30	" + "	0.508
6	7.30	Defibrinated.	0.503

assumption being made that only one such group is so changed. In view of the appreciable differences observed between different bloods, however, and between blood and solutions of recrystallized hemoglobin, it appears preferable to let the publication of such calculations await further data.

SUMMARY.

1. The average buffer value at pH 7.2 to 7.5 of three samples of oxygenated blood from the same horse was found to be $\dfrac{dB(mM.)}{dpH} =$ 25.3, the average value for the same bloods in the reduced condition being 24.4.

2. The buffer values of each blood, the degree of oxygenation being constant, were constant over the pH range 7.2 to 7.5.

3. In the oxygenated blood the hemoglobin was calculated to be responsible for an average of 76.0 per cent of the total buffer value, bicarbonate for 6.9 per cent. In reduced hemoglobin the figures were 73.3 and 9.0, respectively.

4. The difference in buffer value between oxygenated and reduced blood is attributable to a loss of buffer value at this pH when oxyhemoglobin is reduced. The consequent loss to the total buffer value of the blood is only partially compensated by the increase in [BHCO₃] that accompanies the deoxygenation.

5. From the effect of oxygenation and reduction on the buffer value of blood it is estimated that if the molecular buffer value of oxyhemoglobin is 2.64, as found in the preceding paper, the molecular buffer value of reduced hemoglobin is 2.45.

6. At pH 7.3 the decrease in [BHCO₃] per added molecule of oxygen combined with the hemoglobin was found to vary in six bloods from 0.50 to 0.59, indicating that a gram molecule of oxygenated hemoglobin at this pH binds 0.50 to 0.59 more gram equivalents of alkali than does reduced hemoglobin. The variation between 0.50 and 0.59 is probably outside the limit of error in these experiments, and is due to variable factors not yet ascertained in the blood. The ratios observed in blood are lower than those observed in solutions containing only recrystallized hemoglobin and sodium bicarbonate.

7. At a given pH, with varying degrees of oxygenation, the displacement of base from combination with bicarbonate is in simple direct proportion to the amount of oxygen combined. This constancy of the $\frac{dB}{d[HbO_2]}$ ratio was also found with hemoglobin solutions (1).

8. Increase of pH over the range 7.2 to 7.5 increases the amount of alkali in a given blood displaced from combination with bicarbonate (added to hemoglobin) by 1 molecule of oxygen, the increase being at the rate of, approximately, 0.02 equivalent of alkali per 0.1 pH rise.

9. No quantitative differences were detected between defibrinated blood and blood in which coagulation had been prevented by addition of 0.2 per cent of potassium oxalate and 0.1 per cent of sodium fluoride.

BIBLIOGRAPHY.

1. Van Slyke, D. D., Hastings, A. B., Heidelberger, M., and Neill, **J. M.**, *J. Biol. Chem.*, 1922, liv, 481.
2. Joffe, J., and Poulton, E. P., *J. Physiol.*, 1920, liv, 129.
3. Evans, C. L., *J. Physiol.*, 1922, lvi, 146.
4. Henderson, L. J., *J. Biol. Chem.*, 1920, xli, 401.
5. Van Slyke, D. D., *J. Biol. Chem.*, 1922, lii, 525.

ON GLUTATHIONE.

II. A THERMOSTABLE OXIDATION-REDUCTION SYSTEM.

By F. GOWLAND HOPKINS and M. DIXON.

(From the Biochemical Department, University of Cambridge, Cambridge, England.)

INTRODUCTION.

In a previous paper (Hopkins, 1921) an account was given of the isolation from yeast and from animal tissues of a dipeptide containing sulfur. The constituent amino-acids were shown to be glutamic acid and cysteine, and *glutathione* was suggested as a convenient name for the substance. The hydrogen of the $-SH$ group in the cysteine moiety is, as might be expected from many analogies, capable of easy oxidation. Since such oxidation results in the formation of a disulfide group, $-S-S-$, and involves the linkage of 2 molecules, the substance in its oxidized form is no longer a dipeptide in a strict sense, but an association of four amino-acid residues. It would be consistent with the analogy of the cysteine-cystine nomenclature to give a separate name to each form; but it will be convenient, at any rate until the constitution of the substance is completely established, to use the name glutathione alone, and to speak respectively of its reduced and oxidized form. Equilibrium in the living cell would seem to be such that the greater part of the substance present exists in the reduced condition; but oxidation and reduction of the constituent sulfur groups are reversible processes in the tissues, and both forms may at any moment be present.

A full study of the general chemical properties of the substance is in progress. The present communication is concerned with observations meant to elucidate further its relations and functions in the living cell and for the most part only with experimental results which have shown that its activities are associated in an

527

unexpected manner and to a remarkable extent with tissue agencies
which are thermostable.

A word may be said as to the terminology used in the discussion
of the facts. In the case of a system containing a tissue catalyst,
together with substances susceptible to oxidation under the in-
fluence of such a catalyst, it is difficult without detailed knowl-
edge to find criteria concerning the nature of the system which
will fully distinguish between different possibilties suggested in
views that are at present held. If, indeed, the oxidation occurs
only upon access of free oxygen it is legitimate in the present
state of knowledge to assume that an "oxidase" system is in-
volved, of the type pictured by Engler, Bach, Chodat, and others.
If, however, (a reducible substance being present or artificially
provided) oxidation occurs also indirectly under strictly anae-
robic conditions, the suggestion then is that the catalyst acts
either as one determining simultaneous oxidation and reduction
by means of the elements of water, as suggested by Bach; or it
may act rather (the distinction, in certain cases at least, being real)
by determining the transfer of hydrogen from an oxidizable
substance to a reducible substance on the lines suggested by
Wieland (1914). It is impossible without knowing full chemical
details concerning the reaction or reactions involved to decide
between the last two possibilities. For the purpose of the
following discussion it is extremely convenient to assume the view
of Wieland and to employ the related terminology of Thunberg
(1920); to speak, that is, of the substance oxidized as the "hydro-
gen donator" and of the substance reduced as the "hydrogen
acceptor." Under aerobic conditions the acceptor, of course,
may be oxygen itself. When, however, the term hydrogen donator
is used it should be remembered that in any particular case under
consideration the term oxygen acceptor might better correspond
with the facts. No essential confusion, as a matter of fact, arises,
when only general aspects of the phenomena are under discussion,
if these two terms be taken as equivalent.

Into Wieland's conception of hydrogen transport the properties
of the sulfur group of glutathione fit with special readiness. It
will be found, however, that the facts now to be described suggest
that the activities of the dipeptide seem but little concerned with
those of any recognized type of tissue catalyst.

The experiments upon which this paper is based have dealt chiefly with amphibian and mammalian muscle; but less complete observations upon other animal tissues have indicated that the essential statements hold true for these also.

Reduced glutathione, at suitable hydrogen ion concentrations, is oxidized by molecular oxygen, and under similar conditions it freely reduces methylene blue. On the other hand, as was shown in the previous communication, factors are present in the tissues which promptly reduce the oxidized product whenever its concentration is raised above an equilibrium value.

While, therefore, the sulfydryl group of the dipeptide constitutes one at least of the reducing agents of the tissues, its special significance depends upon the circumstance that when it transfers its hydrogen to molecular oxygen, or to such an acceptor as methylene blue, it is itself reduced afresh by normal tissue agencies. A continuous transfer of hydrogen is thus established and, as should be noted, at a velocity much greater than that with which the hydrogen is directly transferred—if it be transferred at all—to the acceptor from the particular sources concerned.

In so far as they relate to the reduction of methylene blue these facts were established by observations described in the first paper. Further experiments have shown that they hold true when the final acceptor is atmospheric oxygen.

An experimental analysis of vital oxidations must involve more than the isolation of intermediary oxidation products. It is clear from what is known that some analysis of the oxidative mechanisms is possible as well as desirable.

The acquisition of interesting facts by the study of tissues before and after they have been merely extracted with water has justified some attempt at analysis on these simple lines. Just as it helped Harden and Young to a recognition of the coferment of alcoholic fermentation, so has this method enabled Meyerhof (1918), to show that two factors, at least, are concerned in the maintenance of respiratory oxidations, and Batelli and Stern (1914) to demonstrate a distinction between soluble and insoluble catalysts in the tissues. Further, by the use of the same simple procedure, involving the removal by washing of the soluble hydrogen donators present, and the restoration of the lost reducing power by the addition of substances of known constitution,

Thunberg has thrown light upon intermediary products as well as upon certain aspects of the oxidative mechanism.

A fresh tissue, when very thoroughly washed, fails to reduce methylene blue or reduces it very slowly. The washing removes *inter alia* the greater part of its content of glutathione. When, under suitable conditions the dipeptide is alone restored to the system, a considerable part of the original reducing power is also restored, just as completely—it should be noted—when the added dipeptide is in the oxidized form (containing, therefore, no mobile hydrogen) as when it is added already reduced.[1] This fundamental fact, demonstrated by observations described in the first paper, has been confirmed by a great number of later experiments. Evidence is supplied in the present paper to show that the dipeptide under these circumstances does not itself act as a primary hydrogen donator. The changes its molecule undergoes are strictly reversible and involve the oxidation and reduction of the sulfur group alone.

Since a preparation of fresh tissue rendered almost incapable of reducing methylene blue by thorough washing with cold water still reduces the oxidized form of the dipeptide and in conjunction with the latter actively reduces the dye, it must presumably still contain sources of labile hydrogen.

It was during an endeavor to ascertain the nature of these by the use of various solvents—among them boiling water and alcohol—that the remarkable stability of a residual tissue system capable of acting in conjunction with the sulfur groups of the dipeptide was first observed.

A *Thermostable Reducing System in Tissues.*

A mass of chopped muscle after it has been very thoroughly washed with cold water may be heated for hours in water at 100°C. or repeatedly extracted with boiling water and finally dehydrated with alcohol, dried *in vacuo*, and ground to a fine powder without losing more than a fraction of that part of its

---

[1] In all our experiments the substance has been used in the oxidized form. Its effect in accelerating reduction or oxygen uptake must then be due to its special relations with the tissue or tissue residue. If added in the reduced form its own reducing power must be allowed for.

reducing power which depends upon the presence of glutathione. Especially is this true if the treatment just described is carried out without contact with oxygen.

A preparation from muscle after undergoing such treatment may fail by itself to reduce methylene blue under any circumstances. But if it be placed in a dilute solution of glutathione and especially if, as in our experiments, it is suspended in a phosphate buffer solution of which the hydrogen ion concentration is around about 7 to 8, and to which glutathione in its oxidized form has been added in amount equal to, say, 10 mg. per gm. of tissue used, a system is established which reduces with rapidity.

The results of an actual experiment may be quoted here; others of a similar nature are more fully described in a later section.

The muscles from two freshly killed rats were passed through a mincing machine and the finely divided tissue was washed ten times with distilled water by shaking in a stoppered cylinder in which the air above the fluid was replaced by nitrogen each time the water was renewed. A portion of the tissue was at this stage reserved, (a), and the rest was heated for 2 hours anaerobically in twice its own bulk of water at 100°C. A portion being again reserved, (b), the rest was boiled out six times with successive quantities of water, (c). At each of the three stages mentioned the muscle residue after the treatment with water was washed with 95 per cent alcohol and dried *in vacuo* over sulfuric acid. All three preparations were then finely powdered and sampled.

With equal quantities of dry tissue and of methylene blue in each case (for details see the Experimental section) the reducing time of (a) by itself was $3\frac{1}{4}$ hours and of (b), $5\frac{1}{4}$ hours; (c) showed no observable reducing power.

Under precisely similar conditions but with the addition of 10 mg. of oxidized glutathione per gm. of dried tissue, (a) reduced in 28 minutes, (b) in 24 minutes, and (c) in 24 minutes (see also Series 2 a). The reducing system which works in conjunction with, and only in conjunction with, the sulfur groupings could have suffered scarcely at all from the severe treatment described, as in each case the velocity of reduction remained practically the same when glutathione was restored to it. The thermostability of a catalytic—or quasi-catalytic—system in the tissue is indeed remarkable.

Whatever the nature of the factors comprised in the system it would, we think, be wholly wrong to look upon them as other than physiological.

It is possible even with fresh unwashed muscle to show that raising the concentration of glutathione accelerates reduction by the tissue, but in this case a relatively large amount should be added. Otherwise, the mechanism with which it is especially concerned is merged in the general reducing power of the tissue and the effect may not be pronounced. After slight washing, sufficient to remove only a part of the dipeptide, the effect of restoring it comes immediately to light. Thorough washing at ordinary temperature removes nearly the whole, and then the effect of supplying it is nearly at a maximum. As we have just seen, the effect is the same after the tissue has been finally submitted to boiling water. At each stage of the treatment factors are shown to be present which reduce only with the coagency of glutathione and by the time the latter has itself been washed away the only remaining factors of this sort left in the tissue prove to be entirely insoluble and thermostable. It does not follow, of course, that these are the only factors having this special dynamic association with the sulfur groupings of the dipeptide. Other hydrogen donators may be soluble in cold water and so be washed away during the removal of the dipeptide itself. But the thermostable factors are quantitatively the more important and once the tissue has been extracted with cold water they seem to be the only ones left. All the evidence points to the circumstance that these thermostable factors act with glutathione in the intact tissue. It is highly noteworthy in this connection that such tissue preparations after thorough extraction with boiling water, can, in the presence (though only in the presence) of glutathione and under controlled conditions, continuously absorb oxygen and yield carbon dioxide. They, in a limited sense at any rate, "respire."

The thermostable tissue residues actively reduce the oxidized form of the dipeptide (as can easily be shown, especially in anaerobic experiments, by the development of the nitroprusside reaction which is given by the reduced form alone). Otherwise the system which "respires," or reduces methylene blue, could not be established. Such residues it would seem must contain hydrogen donators and, presumably, some form of primary catalytic

system for which the glutathione is a coagent. If so, the catalytic system is remarkably stable and the hydrogen donators exceedingly insoluble in water. The factors present in the tissue residue together with the glutathione constitute what may be called the thermostable reducing system of the tissue. It should be understood that this contributes not a negligible fraction, but a quite noteworthy amount to the total reducing power and "respiration" of the tissue.

Effects of Oxygen on the Thermostable System.

The residual tissue system which shows itself to be so highly thermostable, and is activated by glutathione, is sensitive to oxidation. The dry powder when kept in stoppered bottles only very slowly loses its power of transferring hydrogen to the sulfur groups of the dipeptide. On exposure to the air in thin layers this property is lost with somewhat greater rapidity. It is natural to suppose that the change is due to the slow oxidation of the relatively labile hydrogen atoms which in the presence of glutathione are so rapidly activated and transferred to other acceptors.

To hydrogen peroxide the thermostable tissue preparations are exceedingly sensitive. A single observation may be quoted in support of this statement.

A dry muscle powder (2 gm.) which in the presence of glutathione originally reduced methylene blue with high velocity was placed for 15 minutes in 50 cc. of a 0.5 per cent solution of the peroxide and occasionally shaken. It was filtered off and very thoroughly washed with water until neither the washings nor the powder itself showed any trace of peroxide or of active oxygen by the use of the most delicate tests. It was then dried *in vacuo*. All capacity to reduce methylene blue in the presence of glutathione was found to be lost, though a control specimen of the powder, after equally thorough washing with water, was found to have lost none of its reducing power.

Related to the above facts are others which bear directly on the properties of the dipeptide. A thermostable preparation from muscle made on the lines already described and in the form of a dry powder, although not, as already stated, entirely resistant to the influence of atmospheric oxygen, may yet be suspended in, say,

a phosphate buffer solution of pH 7.4, and aerated for some hours at room temperature with a stream of air, without losing more than a small fraction of its powers to reduce methylene blue anaerobically, when subsequently supplied with oxidized glutathione. But suppose a few mg. per gm. of tissue of glutathione (wholly absent from the original thoroughly extracted tissue) be added to the buffer solution *before* the aeration. It will now be found that, after even relatively brief exposure to an air stream at room temperature, the tissue will have wholly lost its power to reduce under anaerobic conditions. This is found to be the case if it·be tested in the presence of the glutathione as originally added before the aeration, or on the other hand, if in order to make the conditions comparable with those of other experiments, it be first washed, dried, and supplied with fresh dipeptide.

Such results indicate clearly that the thermostable extracted residues from muscle contain oxidizable material, or, keeping to the point of view and terminology chosen for the purposes of our discussion, such residues still retain transponible hydrogen for which molecular oxygen, no less than methylene blue, can act as acceptor. The results show also that transport to oxygen is as greatly accelerated by the presence of the sulfur group of glutathione as is transport to methylene blue.

It is clearly desirable that in such a connection quantitative data should be obtained, especially because with oxygen the changes involved can be made to proceed to completion instead of ceasing at an unknown equilibrium point, as in the case of the anaerobic observations made with methylene blue in closed tubes.

For this purpose we employed the well known differential apparatus of Barcroft (see Series 6 and 7 in the next section).

Suspended in phosphate buffer solutions of pH 7 to 8 and shaken with air, such thermostable preparations as we have described are found by themselves to take up oxygen with extreme slowness or not at all. If, however, oxidized glutathione, showing by itself, of course, no oxygen uptake, be added to the suspension fluid in amounts equal to, say, 10 mg. per gm. of dried tissue, a brisk uptake of oxygen occurs. Varying with the amount of glutathione added, and, of course, with the temperature, the time required for the oxidation to reach completion is in the case of 0.5 gm. of tissue from 1 to 5 hours. The total uptake is of the order of

400 c.mm. of oxygen per gm. of dried muscle residue. In a later discussion it will be shown that this represents a by no means unimportant fraction of the normal respiratory uptake of the tissue from which the preparation was made. Significant is the fact that the uptake of oxygen is associated with the production of carbon dioxide in definite amount.

It is difficult to decide what type of material, oxidizable by atmospheric oxygen in the absence of an enzyme, but requiring for its oxidation the aid of glutathione, can thus remain associated with a tissue residue after very thorough extraction with hot water and alcohol. Until this is decided, the full significance of the phenomena described in this paper will not be understood. It is remarkable that the respiratory quotient (if in such a connection the term may be allowed) of the extracted muscle tissue is not constant during the course of the oxygen uptake, but gradually falls from high to lower values. An endeavor to determine what is oxidized in and by the thermostable system is in progress.

In the particular instance of muscle, one point in connection with the oxidations which occur in the thermostable residue, though it is probably a minor one, is worthy of mention.

In the case of yeast, and, almost certainly, in the case of cellular animal tissues such as hepatic and renal tissue, the substance responsible for the nitroprusside reaction given by the fresh tissue, is wholly removable by extraction with water. The reaction is apparently wholly due to the reduced glutathione present. In muscle fiber this is not the case. After the dipeptide has been completely removed from muscle the fibers still yield a strong nitroprusside reaction. The character of the reaction leaves little doubt that it is due to a sulfydryl group, and the suggestion is that one, at least, of the muscle proteins contains cysteine instead of cystine in peptide association. If so, the case is exceptional. As is well known, the proteins of the blood and connective tissue give no reaction, and though it is difficult perhaps so to wash a cellular organ as to obtain an absolutely negative result, the residual reaction is so slight that it may well be due to remaining traces of adsorbed glutathione. This is not so with muscle.

In any case the fixed $-SH$ group in the muscle fiber can exert none of the functions subserved by that present in the soluble dipeptide. The extracted residues which give in all cases a

strong nitroprusside reaction are, as we have seen, by themselves
quite incapable of reducing methylene blue or of taking up oxygen.
An interesting phenomenon in which both the "fixed" sulfur
group and the sulfur group of glutathione are concerned may be
easily observed. The sulfydryl as it exists in the tissue residue is
itself scarcely at all autooxidizable. When the muscle residue is
suspended in water or buffer solutions and the suspension thor-
oughy aerated, or when it is shaken in a Barcroft apparatus,
as in the experiments just described, the nitroprusside reaction
survives almost indefinitely. If, however, glutathione be added
to the solution before the aeration, the fixed $-SH$ is oxidized and
the strong nitroprusside reaction originally given by the tissue
residue rapidly disappears. One of the tissue constituents of which
the oxidation is catalyzed by the sulfur group of glutathione is,
therefore, itself a sulfur group.[2] It is clearly not the only con-
stituent so oxidized because the "respiration" of the thermostable
residue involves always the production of carbon dioxide.

Oxygen Uptake by Washed but Unheated Tissue.

In discussing the influence of glutathione in promoting oxygen
uptake we have hitherto, for convenience in discussion, referred
chiefly to its effect upon heated tissue residues. Since it acts
with these it would be expected to act with tissues simply
washed. It undoubtedly does so; but for the clear display of this
property certain experimental conditions must be secured. If
muscle, after thorough washing with cold water, but without
further treatment, be supplied with glutathione and shaken
in a Barcroft apparatus under conditions similar to those already
described, an uptake of oxygen is observed; but it is slow and ir-
regular. If, however, the washed residue be first extracted with
alcohol, or if it receive only a very brief preliminary treatment
with alcohol—the alcohol being then completely removed by fur-
ther washing with water—the moist residue will now, in the
presence of glutathione show a steady uptake of oxygen, which if
due allowance be made for any variation in the water content,

[2] A paper by Dixon and Tunnicliffe on the conditions controlling the
oxidation of -SH groups, which will shortly be published from this labo-
ratory, contains results which bear upon the above phenomenon.

will be found equal to the uptake of a heated preparation. This correspondence is easily shown if the unheated and heated preparations are both employed in the form of a dry powder. There seems to be little doubt that the effect of the preliminary treatment is on permeability. Heat and treatment with alcohol seem to influence this on similar lines. It is noteworthy that although, as we have repeatedly stated, a washed residue without treatment establishes with the dipeptide a system which actively reduces methylene blue yet even in this case preliminary washing with alcohol or heating to 100°C. increases the velocity with which the system reduces.

The Stability of Glutathione.

It is important for proper appreciation of the part played by the dipeptide in the phenomena we have described, to realize that when its addition evokes activity in inactivated tissue preparaparations, this is not because it, itself, acts as a primary hydrogen donator or oxygen acceptor. Its molecule is not oxidized in the sense that, say, succinic acid is oxidized by isolated tissues, or in the sense that the various substances studied by Thunberg are oxidized anaerobically by washed tissue in the presence of methylene blue. As Thunberg found that glutamic acid, one of its constituent amino-acids, is so oxidized, it might be expected that the dipeptide would suffer the same fate. This is not so. To judge, at least from experiments with excised tissue, its molecule as a whole is stable. In its case hydrogen attached to carbon is not mobilized, but only that attached to sulfur. The reversible change from the sulfydryl to the disulfide grouping is the essential happening.

In proof of this statement we rely in particular upon the study of equilibria. When in anaerobic experiments with washed tissue and methylene blue a substance which acts as a primary hydrogen donator is added to the system (succinic acid, for instance, as a precursor of fumaric acid) the amount of methylene blue reduced when equilibrium is reached will naturally be proportionate to the amount of donator supplied. This is not the case when glutathione is in question. The final equilibrium in the system is quite independent of the amount present. This is clearly seen from the results of a series of observations (Series 3) in the next

section. An 8-fold increase in the concentration (the weight of tissue being the same) is seen to have no effect whatever upon the amount of methylene blue finally reduced. On the other hand the velocity of reduction increases with the concentration of the dipeptide. Such results are consistent with the properties of a substance concerned with hydrogen transport on the lines assumed in the course of this discussion, but the facts could not apply to one acting as the primary hydrogen donator.

Equally definite evidence is obtained from a study of the effects of varying the concentration upon the oxygen uptake of a tissue residue. The results of Series 7, for instance, show that a 4-fold increase in the concentration of glutathione, while increasing the velocity of uptake, made no difference to the amount of oxygen ultimately taken up.

It may be well to point out that tissue preparations have no power to oxidize glutathione as the result of such surface activity as that involved in the oxidation of amino-acids by charcoal.

Warburg[3] has recently demonstrated that various substances, including certain amino-acids, such as leucine, tyrosine, and cystine, are completely oxidized when their solutions are shaken with blood charcoal in the presence of air. No such property as this belongs to the tissue residues either when moist or when they have been dried and ground to a fine powder. We could discover at least no trace of ammonia or carbon dioxide production when leucine or tyrosine solutions were shaken for long periods in the presence, e.g. of a dried muscle powder supended in the fluid. Warburg obtained striking results with cystine. In this case the behavior of a tissue residue differs entirely from that of charcoal. It reduces the cystine to cysteine and then follow effects qualitatively similar to those observed with glutathione.

The Relation of Glutathione to other Respiratory Systems.

The total reducing power of any tissue represents doubtless the sum of the activities of several different types of chemical mechanism. This is equally true of its total capacity for oxygen consumption. It is becoming evident, indeed, in a greater number of cases than was previously recognized, that a catalytic agency which

---

[3] Warburg, O., and Negelein, E., *Biochem. Z.*, 1921, cxiii, 257.

promotes a respiratory process may be one which under other conditions is responsible for reductions.

A cold water extract of a tissue contains those oxidizing agencies which are at present known more particularly as "oxidases"— the alcoholase of Batelli and Stern, catalysts of the tyrosinase type, purine oxidases, and the like. To these, or to some of them, and to the simultaneous presence of substances upon which they act (individually present in minute amounts) a cold water extract of a tissue owes part of its reducing power. Morgan, Stewart, and Hopkins (1922), for instance, have shown that tissue extracts oxidize xanthine and hypoxanthine anaerobically in the presence of methylene blue, the latter acting as the hydrogen acceptor in what is probably a hydrolytic oxidation-reduction process. There is no good reason to suppose that the catalyst here concerned is distinct from the xanthine "oxidase" of other authors. Its action has usually been studied under aerobic conditions when oxygen instead of methylene blue becomes the hydrogen acceptor.

The point, however, that we wish to emphasize here is that so far as we have been able to discover, glutathione exercises no functions in connection with oxidizing agencies of this type. If, at any rate, it be added to a cold water extract of muscle the velocity with which such an extract reduces methylene blue is, at most, but slightly accelerated. The sulfur group seems to have no relation with the soluble tissue enzymes.

As is well known, Batelli and Sterns have distinguished from the soluble oxidases certain insoluble agencies in the tissues which they have called oxydones. The activities of these are, so far as we know at present, associated with the tissue structure as left after extraction with cold water. Such agencies may represent something more than the equivalent of specific enzymes; they may comprise surface effects or other such factors. It is abundantly certain, however, that animal tissues after the most complete extraction with cold water retain unstable oxidation-reduction mechanisms possessed of marked activity. Their activity can be demonstrated either by the occurrence of direct oxidation in the presence of air or by indirect anaerobic oxidations brought about in the presence of such a substance as methylene blue. The systems in question are thermolabile and characteristically unstable. It is by means of mechanisms of this class that excised tissues so

readily oxidize succinic and citric acids (Thunberg, and Batelli and Stern) and probably by similar means many, if not all, of the substances in the extended list recently studied by Thunberg are oxidized.

With these insoluble but unstable catalytic mechanisms, as with the soluble catalysts, the sulfur constituent of the cell would again seem to have no concern. Studying at any rate the typical cases of the oxidation of succinic and citric acids, or rather, the power of these substances to act as oxygen acceptors (or hydrogen donators) in the process of methylene blue reduction, we have found that glutathione exerts in these connections no influence. The washed tissue with little or no reducing power of its own reduces actively when succinic acid is supplied. If to this combination glutathione is also supplied the effects observed are merely additive (Series 4). It would seem indeed that the power to reduce the disulfide $(-S-S-)$ group to the sulfydryl group $(-SH)$ and thus to establish with glutathione a system which can transport hydrogen to methylene blue, or on the other hand a system which is autooxidizable, is to a peculiar degree attached to agencies which are not enzymes and are neither extracted nor destroyed by boiling water. Furthermore, we have so far been unable to prove with certainty that any known soluble tissue constituent or metabolite is oxidized under the influence of the system constituted by the thermostable residue plus glutathione. No pure substance yet tried has by its addition to the system increased the amount of methylene blue it can reduce or added to its total oxygen uptake. Nevertheless, we have so fractionated and concentrated aqueous muscle extracts as to obtain preparations which do affect equilibrium in the system to a marked degree.

The Relation of Glutathione to the Atmungskörper of Meyerhof.

There seemed from the first to be grounds for supposing that glutathione with its active sulfur group might be identical with the "respiratory substance" (Atmungskörper) of Otto Meyerhof (1918), or, at least, form part of a chemical system described under that name. Since the first brief account of the properties of the dipeptide was published this possibility has occurred to others.[4]

[4] Compare Dakin, H. D., *Physiol. Rev.*, 1921, i, 403.

3 years, indeed, before glutathione was isolated, Meyerhof had fully considered the possibility that his Atmungskörper might be a substance carrying the $-SH$ group, and he endeavored to test the matter experimentally. He points out in a highly interesting paper that, broadly speaking, there is a parallelism between the intensity of the nitroprusside reaction, as given by a yeast extract or "Kochsaft" and the activity of such an extract in restoring respiratory activity to washed acetone yeast. In his experiments cysteine appeared to have no such effect, but in order to test that matter further he studied the influence of thioglycollic and thiolactic acids. He found as a matter of fact that when a yeast residue, inactivated by washing, was suspended in a neutral or slightly acid solution of either of these substances, an uptake of oxygen occurred, considerably in excess of what would be required to oxidize the added thio-acid to the disulfide form.

The properties of the substances employed by Meyerhof were, however, such as to make the precise meaning of the experimental results somewhat obscure. The author found, for instance, that if thioglycollic or thiolactic acid be added to the tissue in the form of the corresponding disulfide derivative, neither substance promotes oxygen transport. The reason must be that the tissue does not reduce the $-S-S-$ group of these compounds.[5] The processes involved do not seem to be in any obvious sense reversible, therefore, and it is difficult under the circumstances to understand how the $-SH$ group of these thio-acids can promote continuous oxygen transport.

On the other hand, as we have many times pointed out, the disulfide form of glutathione is freely reduced by tissues, or washed tissue preparations, a characteristic which in this connection seems to be highly important.

Meyerhof noted another quality in the sulfur group of thioglycollic acid (the substance most fully studied) which renders remarkable its ability to promote under the conditions of his experiments the uptake of oxygen by a washed tissue.

In neutral or slightly acid solutions (pH 6 to 7) the $-SH$ group of this acid is stable and not autooxidizable, yet it is only in solu-

[5] Since the above was written we have ourselves found that dithiodiglycollic acid is, as a matter of fact, reduced by washed muscle and washed acetone yeast. The addition of these substances does promote oxygen transport, though much less efficiently than glutathione.

The above remarks, based upon Meyerhof's own findings, therefore lose their point.

tions with this range of pH that the substance promotes respiration of the washed tissue. In more alkaline solutions it is itself autooxidizable, but then does not promote respiration, because as soon as it is oxidized to the disulfide form its relations with the tissue cease. Here again the properties of the natural constituents differ in a significant sense. The $-SH$ group of glutathione is autooxidizable in neutral solution and it promotes the uptake of oxygen by a washed tissue within just that range of pH in which it is itself autooxidizable, and also capable of reduction by the tissue. It would seem at first sight as though the sulfur group of the natural constituent is more likely to possess the functions generally pictured for the Atmungskörper than that of thioglycollic acid. Nevertheless, the facts brought forward in previous sections of this paper show that it is impossible to identify the dipeptide with any substance supposed to stimulate respiratory processes in general. Meyerhof, having observed that the influence of the sulfur group of thioglycollic acid was but little affected when the tissue residue (washed yeast) had undergone heating to 100°C.,[6] pointed out that this must constitute the most important distinction between the thio-compound and an agent of the coferment class, to which, *ex hypothesi*, the Atmungskörper belongs.

From our own standpoint, as maintained throughout the present paper, the fact that the activities of the sulfur group seem to be unrelated to enzyme activities does not remove biological significance from the former; but enough has been said in previous sections to show that there are processes involved in the sum total of respiratory activity with which it has no direct concern.

Meyerhof's fundamental observation which led to the conception of the Atmungskörper was the activation of washed tissues by a tissue "Kochsaft." It is not unimportant to consider all that is involved in this phenomenon. A considerable part at least of the oxygen uptake which occurs is due to the fact that relatively insoluble catalysts present in the washed tissue are again supplied with soluble oxygen acceptors from which the original washing divorced them.

[6] When we first observed that glutathione worked with heated tissue residues we were unaware that Meyerhof had 3 years before (1918) made the above observation with thioglycollic acid.

Any of the numerous substances proved by Thunberg, by Batelli and Stern, and by Meyerhof to be oxidized by a washed tissue, or shown in the later work of Thunberg to be capable of acting as hydrogen donators, may constitute oxidizable material in a Kochsaft. The concentration of any one of them will admittedly be very low, but now that we know they may be numerous it must be recognized that, collectively, their oxidation may account for a considerable oxygen uptake. As already stated the oxidation of such substances—as represented by succinic, citric, and certain amino-acids—by thermolabile catalysts in the tissue is unaffected by the presence or absence of glutathione. There is, on the other hand, no evidence to show that these particular oxidations require the presence of a soluble coagent of any sort.

Some observations of our own, concerned with animal tissues only, have a bearing on these considerations. We have prepared on Meyerhof's lines from perfectly fresh tissues very active samples of Kochsaft and have found that such preparations, if not exposed to the air, retain their activity for long periods. On the other hand we found that if the tissues from which they were prepared were allowed to stand before extraction, the extracts made from them grew progressively less active as the time allowed for survival or postmortem changes in the tissue was made longer. Such results suggest the disappearance of oxidizable material during the course of survival events rather than the destruction of a coferment. We are, of course, not suggesting that a Kochsaft contains no substance of such a type. Meyerhof's classical studies have shown that it does. We find, however, one difficulty in connection with the present discussion. It seems to be commonly assumed, though not by Meyerhof himself, that the Atmungskörper is a single substance stimulating respiratory processes as a whole. If that were so it would certainly be an agent quite distinct from glutathione. If, on the other hand, the name may be taken to connote the activities of two or more substances each related to some particular aspect of respiratory oxidations, then glutathione shows itself to be one of these; its activity being more particularly, though not exclusively, related to respiratory factors (whatever they may prove to be) which remain intact in a tissue when it has been heated.

A direct experimental demonstration, however, even of this established fact is not easy by the use of Kochsaft preparations. The concentration of glutathione in a Kochsaft may be considerably increased with relatively little effect upon its activity when added to a washed tissue. This, we think, is partly because the effect is at first swamped in the progress of rapid oxidations which, as we have just pointed out, proceed without the influence of any soluble coagent. There is, however, a further circumstance of importance. The physical condition of a washed tissue is such that part of its uptake of oxygen which depends specifically upon the presence of glutathione is slow (see above). On the other hand, the preliminary treatment with alcohol which, as we have seen, so efficiently promotes the combined activity of the tissue and the sulfur group renders the former entirely inactive towards the other constituents of a Kochsaft. Such facts as these require further study for their elucidation.

<div style="text-align:center">EXPERIMENTAL.</div>

Section 1. Anaerobic Experiments.

All our measurements of reduction velocity were made under anaerobic conditions. The tissue preparations were suspended in a phosphate buffer solution contained in test-tubes which could be evacuated. The tubes employed (somewhat similar to those used by Thunberg) are $4\frac{1}{2}$ inches long with a diameter of $\frac{3}{5}$ inch. A short side tube projects from a tubular neck which is ground to receive a hollow glass stopper perforated by a hole at the level of the side tube. The tubes are evacuated when the hole has been made to correspond with the opening of the side tube, and if a suitable stopper grease be used a vacuum is maintained almost indefinitely after the tube has been closed by turning the stopper. For the purpose of our experiments it was found sufficient to evacuate the tubes by means of a good filter pump. In order, however, that the small residual oxygen tension should be identical in any series involving a comparison, branch connection tubes were provided so that six or more of the test-tubes could be connected at once with the same pump, and so exhausted to exactly the same degree.

All the determinations of reduction time recorded in the following tables were made in a phosphate buffer solution containing

primary potassium phosphate and secondary sodium phosphate (Sörensen). Almost invariably the pH of the system was 7.6. The glutathione, which is itself markedly acid, was dissolved in a somewhat more alkaline phosphate mixture (e.g., of pH 8) and the solution then adjusted to 7.6, the volume being also adjusted to yield a known concentration of the dipeptide. The required amount of a tissue preparation was first weighed into a reduction tube. It was then covered with a known volume of the buffer solution, either plain or containing glutathione (or other substance to be tested) in known amount. The final volume and the pH were always made the same in each tube of a series under comparison. The methylene blue solution was added last and the tube or tubes immediately evacuated. After exposure to the tension of the pump for a standard time (usually 3 minutes after the manometer registered a steady pressure) the tubes were closed and transferred to a bath at 37°C. In most of the experiments which were concerned with a comparison of reduction velocities very small amounts of methylene blue were employed (0.2 to 0.3 cc. of 1:5,000). The observations were thus shortened without any loss of significance in the results. When final equilibria were to be determined stronger solutions of the dye were employed. The end-point taken coincided with complete decolorization of the tissue mass as well as of the supernatant fluid.

The observations recorded in the tables which follow represent only a small proportion of the consistent series which have been made. The records occupy much space. We have selected, therefore, results which are typical of the average.

Series 1. Tissue Extracted with Cold Water Only.

The muscle (or other tissue) after excision was finely chopped with sharp scissors until a homogeneous almost pasty mass resulted. It was then transferred to a stoppered cylinder and well shaken with successive quantities of distilled water. The ease with which the glutathione contained in the original tissue, together with other reducing factors, is removed varies somewhat with the tissue employed. In the case of frog muscle six successive washings with 50 to 60 times its weight of distilled water will usually yield a preparation with very small residual reducing power. In the case of fresh muscle from the rabbit or rat we have usually em-

ployed ten washings. After each successive washing the tissue
mass was squeezed in a linen cloth. If its reducing power was to
be studied in the moist condition the mass was finally pressed
between filter papers and the amount required for an experiment

TABLE I.

Washed tissue preparation.	Buffer solution (pH 7.6).	Oxidized gluta- thione.	Methylene blue 1:5,000.	Reduction time.	
gm.	*cc.*	*mg.*	*cc.*	*hrs.*	*min.*
(a) Frog muscle................0.5	5	0	0.3	6+	
0.5	5	2	0.3	0	55
0.25	5	2	0.3	1	35
0.5	5	4	0.3	0	31
(b) Rat muscle................0.6	3	0	0.2	0	50
0.6	3	2	0.2	0	25
0.3	3	0	0.2	1	40
0.3	3	2	0.2	0	59
0.6	3	4	0.2	0	14
(c) Rabbit muscle.............0.5	3	0	0.3	2	40
0.5	3	4	0.3	0	18
(d) Ox muscle................0.5	3	0	0.3	5+	
0.5	3	4	0.3	0	21
(e) Ox muscle as dry powder......0.2	3	0	0.3	6	20
0.2	3	2	0.3	0	40
0.2	3	4	0.3	0	18
(f) Rabbit kidney.............0.5	3	0	0.3	5	30
0.5	3	4	0.3	0	16
0.5	3	8	0.3	0	9

The few results recorded in Table I are sufficient to show the restoration
of reducing power to washed tissue which results when glutathione is
supplied. The latter, which was always employed in the oxidized form,
has itself no reducing power. In all cases except (e), the tissue prepara-
tion was weighed moist. Prepared as described above the tissue residues
have a water content which is somewhat higher than that of the original
tissue. The preparations were made immediately after the death of the
animal except in the case of ox muscle, (d) and (e), which was prepared
from butchers' meat (rump steak).

weighed out as quickly as possible into the tubes. Occasionally the washed tissue was dried *in vacuo* over sulfuric acid and finally ground to powder.

Series 2. Tissue Residues after Extraction with Boiling Water.

After the activity (in conjunction with glutathione) of thermostable tissue residues was first observed we found it desirable for the purpose of comparisons to deal with dry powdered preparations prepared on standard lines. When, for instance, the activity of a preparation obtained by simply washing the original tissue is to be compared with that of the same tissue after heating to 100°C., or after extraction with hot water, a change in the physical condition of the preparation may somewhat affect the velocity with which it reduces methylene blue or takes up oxygen. If both preparations are dried, reduced to powder, and properly sampled—treatment which does not at all reduce the capacity to work with glutathione—the comparison is more accurate.

The tissue may be first thoroughly extracted with cold water and subsequently heated to 100°C. and dried. It is best, however, to arrest survival processes by heating immediately after removal from the animal. Our standard preparations which in the case of muscle show remarkable uniformity of behavior were made as follows.

The tissue is put through a mincing machine and immediately thrown into about thrice its bulk of boiling water. After cooling it is squeezed in a linen cloth and then quickly extracted six times with successive small quantities of boiling water. It is finally washed with a small quantity of alcohol, dried over sulfuric acid *in vacuo*, powdered, and sampled.

All the tissue preparations in Table II were used in this form.

Series 2a.—The two experiments described under this head illustrate how small is the effect upon the activity of a tissue with glutathione when simple washing is followed by extraction with hot water.

In each case the muscle was first washed ten times with 60 times its weight of distilled water under anaerobic conditions (nitrogen). A portion of this washed, but unheated, preparation was reserved for experiment. The remainder was placed in a flask just covered with water and then heated on the water bath

for 2 hours in an atmosphere of nitrogen. A portion was again reserved at this stage. The rest was finally extracted six times with boiling distilled water. A portion of the residue at each stage was washed once with 96 per cent alcohol, dried in a vacuum desiccator, powdered, and sampled. Preparation A was from ox muscle (beef); Preparation B was from the muscle of a recently killed rabbit.

TABLE II.

Tissue preparation weighed in form of dry powder.	Buffer solution (pH 7.6).	Oxidized gluta-thione added.	Methylene blue 1:5,000.	Reduction time.	
gm.	*cc.*	*mg.*	*cc.*	*hrs.*	*min.*
(a) Frog muscle....................0.2	3	0	0.2	24+	
0.2	3	4		0	22
(b) Rat muscle.... ....0.2	3	0	0.2	12+	
0.2	3	2	0.2	0	17
(c) Rabbit muscle.................0.2	3	0	0.2	24+	
0.2	3	4	0.2	0	18
(d) Rabbit muscle, another prepa-ration......................0.2	3	0	0.2	20	
0.2	3	4	0.2	0	12
(e) Ox muscle.....................0.2	3	0	0.2	5	36
0.2	3	2	0.2	0	30
Same preparation heated fur-ther..........................0.2	3	0	0.2	9+	
0.2	3	2	0.2	0	28
(f) Rabbit muscle.................0.5	3	0	0.2	7	20
0.5	3	2	0.2	0	15
0.5	3	8	0.2	0	4

In each observation the tissue was suspended in a phosphate buffer solution of pH 7.6. The dipeptide added was dissolved in a similar fluid with pH adjusted to the same figure. The total volume in each tube was the same ($2\frac{1}{2}$ cc.). Dried tissue 0.2 gm., glutathione 4 mg., methylene blue 0.2 cc. (1:5,000). The figures are the times taken for complete reduction.

Although the residual reducing power of the tissue by itself became zero after final treatment the reduction with glutathione remained as active as after simple washing. When the washed tissue is tested in its original moist condition heating to 100°C. always markedly increases the velocity with which it reduces in the presence of the dipeptide.

Series 3.—Experiments were carried out to determine the effect of varying the concentration of glutathione upon the total amount of methylene blue reduced when equilibrium is attained. The observations were made in evacuated test-tubes on the lines already described. The tissue employed was muscle in the form of the thermostable preparation, dried and powdered. The dipeptide was dissolved in a phosphate buffer and the solution finally made to contain 8 mg. in 1 cc. at pH 7.6.

TABLE III.

	Washed anaerobically.		Heated 2 hrs.		Extracted with hot water.	
	hrs.	*min.*	*hrs.*	*min.*	*hrs.*	*min.*
Tissue alone.						
Preparation A........	3	16	5	16	20+	
" B........	2	8	2	30	20+	
Tissue plus glutathione.						
Preparation A........	0	30	0	25	0	26
" B........	0	21	0	19	0	19

For each experiment four series of tubes were prepared, every tube containing 0.2 gm. of dried tissue. The first series contained in each tube 2 mg. of glutathione, the second series 4 mg., the third 8 mg., and the fourth 16 mg. In each series the amount of methylene blue (1:1,000) was increased in successive tubes by increments of 0.1 cc. The total volume of fluid was made in every tube the same, variations in the quantity of glutathione solution being balanced by suitable additions of buffer solution, and variations in the methylene blue solution by additions of distilled water. To each tube 1 drop of chloroform was added. All, after evacuation, were placed in a bath at 35°C. and left over night. When equilibrium was reached the point of transition from a tube in which the contents still showed a faint blue color to one which was colorless was noted in each series.

The wide variations in the concentration of the dipeptide had little or no effect upon the equilibrium point.

In one experiment with rabbit muscle the contents of the tube in the first series containing 1.2 cc. of methylene blue were faintly but definitely blue. The next tube (1.1 cc.) was colorless. This point of transition was identical in each of the four series; with 2 mg. of glutathione it was exactly the same as with 16 mg., though in the latter case equilibrium was reached much sooner. The tissue preparation by itself showed no reducing power.

In a second experiment carried out on similar lines with a preparation of rabbit muscle, made with special precautions to avoid oxidation during the earlier stages of washing and extraction, the point lay between 1.7 and 1.8 cc. of methylene blue and was again identical in each series.

In an experiment with frog muscle it lay in each series between 0.8 and 1.0 cc. Sometimes in such experiments the results were not quite so sharp, it being difficult to distinguish with certainty between two adjacent tubes in a series. Even an 8-fold increase in the concentration of the dipeptide was never observed, however, to make an appreciable difference in the equilibrium point, whereas, no matter what the concentration of the associated dipeptide, an increase in the amount of tissue produced always a proportionate increase in the amount of methylene blue finally reduced.

Such results show that glutathione cannot itself be a primary hydrogen donator in the reducing system which it establishes with the tissue residue (see also Series 8).

Series 4.—A number of experiments have been carried out to discover whether known metabolites can act as primary hydrogen donators when added to the system constituted by a washed tissue residue and glutathione; in other words to decide whether the anaerobic oxidation of such substances by a tissue residue in the presence of methylene blue is accelerated by the sulfur group of the dipeptide. In general, negative results have been obtained; but the enquiry is by no means complete. It will be sufficient for the purposes of the present paper to give certain illustrative cases.

The oxidation of succinic and citric acids by tissue catalysts has been much studied, and Thunberg has shown that these acids

act typically as hydrogen donators in the reduction of methylene blue by washed muscle.

The following observations show that the presence of glutathione is without effect upon the velocity of this reduction.

Preparation A was one of rat muscle washed ten times with distilled water under conditions made as anaerobic as possible. It was not heated and was weighed moist. Preparation B was rabbit muscle extracted six times with boiling water, washed with alcohol, dried, and powdered. The succinic and citric acids were dissolved in phosphate buffer solution adjusted to pH 7.6. The final solution was one-tenth molar, and 1 cc. was used for each observation. The glutathione (oxidized form) was also in 7.6 buffer solution, 1 cc., containing 4 mg., being employed. Adjustments to secure equal volumes were made with plain buffer solution also at pH 7.6. Methylene blue 0.2 cc. (1:5,000). Observations in evacuated test-tubes as before. The figures in Table IV are times taken for complete reduction at 35°C.

TABLE IV.

	Preparation A 0.5 gm.		Preparation B 0 2 gm.	
	hrs	min.	hrs.	min.
Tissue alone....................................	5	26	24+	
" with glutathione......................	0	34	0	26
" " succinic acid.....................	0	33	24+	
" " glutathione and succinic acid......	0	16	0	2S
" " citric acid.......................	0	55	24+	
" " glutathione and citric acid........	0	21	0	2S

In the unheated Preparation A the catalysts responsible for the oxidation of succinic and citric acids (the oxydones of Batelli and Stern) are, of course, intact. It will be seen from the table that glutathione has no apparent effect upon the anaerobic activity of these. The reduction velocity induced when glutathione is added to either acid represents only the combined effect of each constituent acting with its own proper mechanism. In the case of the heated Preparation B the oxydones are destroyed. The presence of succinic or citric acid has in this case no effect upon the reduction velocity induced by glutathione alone.

Many experiments of the above type have been made, using a variety of substances in the place of the above acids. Only in the case of lactic acid has evidence so far been obtained to show that the anaerobic oxidation of the substance is influenced by the

presence of the dipeptide. Even in this case the requisite conditions are somewhat difficult to define. The experiments which bear upon the matter will form the subject of a separate paper.

Section 2. Observations on Oxygen Uptake.

Series 5.—The following observations show on other lines that what we have called thermostable tissue residues contain oxidizable materials. The oxidation of these by atmospheric oxygen is however, exceedingly slow, except when glutathione is present. It then becomes characteristically rapid.

A thermostable preparation of rabbit muscle made on the lines already described and in the form of a powder was found, as in all cases, to show by itself little or no power of reducing methylene blue. In the presence of glutathione it reduced freely.

TABLE V.

	Reduction time.	
	hrs.	*min.*
Original preparation..	0	18
Aerated alone (6 hrs.)......................................	0	22
" in the presence of glutathione (3 hrs.)..............	5	14

1 gm. of the powder was suspended in a phosphate buffer solution (pH 7.6) and the suspension briskly aerated at room temperature for 6 hours. A second gm. was similarly suspended but in the presence of 10 mg. of glutathione. This was aerated also at room temperature and side by side with the above, but for 3 hours only. Both samples were filtered off, thoroughly washed side by side until the second sample was again free from dipeptide, and then dried *in vacuo* so as to be comparable with the original powder. The portions thus treated were then compared in respect of their residual reducing power with the original preparation. The figures given in Table V are for 0.2 gm. of the preparation, 4 mg. of oxidized glutathione being supplied in each case. Conditions as in previous experiments with methylene blue.

The results in Table VI were obtained in the case of rat muscle, prepared and treated on lines similar to the above. The aeration was carried out at 35°C.

Such experiments show that the thermostable preparations contain hydrogen donators which are quickly oxidized by molecular oxygen in the presence of the sulfur group of the dipeptide, but which show considerable resistance to oxidation in its absence.

Series 6.—The oxygen uptake of thermostable tissue residue in the presence of glutathione was determined quantitatively in the familiar differential apparatus of Barcroft. A weighed quantity of the preparation under study, suspended in a measured quantity of a buffer solution containing a known amount of glutathione (pH usually 7.6), was placed in one flask of the apparatus. KOH for the absorption of CO_2 was present in the chamber provided. In the second flask was placed a quantity of distilled water or buffer solution equal to that in the first. The apparatus after proper adjustment was then shaken in a water bath at known temperature until cessation of movement in the manometric fluid showed that the oxygen uptake was complete. Control observations (*e.g.* with the tissue preparation in the absence of glutathione) were usually made in a second apparatus shaken side by side with the first. Apart from the ordinary careful

TABLE VI.

	Reduction time.	
	hrs.	*min.*
Original preparation...................................	0	17
Aerated alone ($1\frac{1}{2}$ hrs.)................................	0	24
" in the presence of glutathione ($1\frac{1}{2}$ hrs.)............	∞	

calibration of the instruments it is then important if velocity of uptake is under study, to make sure that they yield strictly comparable results. In the case of a system such as that constituted by a suspension of a tissue a relatively small difference in the shape of the flasks may appreciably affect the rate of uptake. It is in any case best to use flat bottomed flasks shaped so as to secure a relatively large surface for the suspension fluid.

The observations recorded in Table VII show the total oxygen uptake of various thermostable preparations in the form of a dry powder suspended in a buffer solution. It should be observed that as the dipeptide was always added in the oxidized form there was no need to allow for oxygen taken up by preexisting $-SH$ groups. If any such experiment be stopped before the uptake of oxygen by the system is complete a nitroprusside reaction can always be observed in the suspension fluid, showing that the tissue preparation has reduced the disulfide group. When the oxygen

uptake has ceased no reaction is obtained. The hydrogen donators in the tissue are then exhausted and the reduced dipeptide itself reoxidized.

Series 7.—Experiments were done to determine the carbon dioxide production which is associated with the oxygen uptake of thermostable tissue preparations. It is easy to demonstrate that CO_2 is actually produced and that its production with any appreciable velocity is dependent upon coagency of glutathione. If a gram or two of a dried preparation be suspended in water, or in a buffer solution, and if the suspension be then briskly aerated with a stream of air which is subsequently led through a solution of barium hydroxide, scarcely any visible clouding will be seen

TABLE VII.

Tissue preparation.	Gluta-thione present.	Tem-pera-ture.	Total oxygen uptake.	Time for complete uptake.		Uptake per 1 gm. tissue prepa-ration.
gm.	*mg.*	°C.	c.mm.	hrs.	min.	c.mm.
Rabbit muscle..............0.5	0	20	6	4		12
0.5	4	20	209	4		418
0.2	8	20	82	1½		410
Rabbit muscle. Another preparation...............0.5	0	20	8	3	10	16
0.5	8	20	205	3	10	410
Rat muscle.................0.5	0	20	0	3	20	0
0.5	4	20	211	3	20	422
0.5	8	35	201	1	50	404

even in the course of several hours. If, however, a few milligrams of glutathione be added to the suspension the baryta solution begins to cloud almost at once. This, as other experiments show, is not due to the oxidation of the dipeptide itself.

To measure the CO_2 production two separate sets of the Barcroft apparatus, known to give corresponding results, were employed simultaneously. In one of these potash was placed in the flask containing the tissue, whereas in the other apparatus no potash was present. Exactly similar quantities of tissue preparation, glutathione, and buffer solution were placed in each and the two were shaken side by side. The difference shown in the respective manometer readings measures the CO_2 production.

To save space the results of one experiment only will be given (Table VIII). They are typical of others obtained except that in different experiments the actual rate with which the ratio $\dfrac{CO_2}{O_2}$ falls off may vary somewhat.

Series 8.—Several experiments were made to determine the effect of varying the concentration of glutathione upon the total uptake of oxygen by the system constituted by a thermostable tissue residue and the dipeptide. The results were entirely consistent in showing that wide variations in the concentration, while of course, influencing the velocity of uptake, have no effect upon the total amount of oxygen finally absorbed. Such results confirm those obtained with the methylene blue technique (Series 3) in showing that the dipeptide is not itself oxidized.

TABLE VIII.

Rabbit muscle, dry preparation, 0.2 gm., glutathione 8 mg., buffer solution 3 cc., bath at 35°C.

	Oxygen absorbed.	CO_2 produced.	$\dfrac{CO_2}{O_2}$
	c.mm.	c.mm.	
First 20 minutes..................	27.2	27.2	1.0
Next 30 "	33.2	20.0	0.6
Final 35 "	12.1	4.0	0.33

We give, in the form of curves of oxygen uptake, the results of a typical observation of this kind. It will be seen that a 4-fold increase in the concentration of the dipeptide (the amount of tissue remaining the same) while it about doubled the initial velocity of uptake had no effect upon the total amount of oxygen absorbed.

DISCUSSION OF RESULTS.

Considered from any standpoint that is familiar, the circumstance that a not inconsiderable part of the respiratory activity of a tissue can depend upon factors that are completely thermostable must appear surprising. The conclusion that such factors function in life is one which seems, at first sight, difficult to accept. We think, nevertheless, that if the evidence be duly considered this conclusion becomes inevitable.

With regard to the substance glutathione it would seem that the properties of its sulfur group are such that once the presence of the dipeptide in a tissue has been shown, a recognition of the fact that it exerts a real influence in the processes of reduction and oxidation as they occur in that tissue follows almost as a corollary.

The $-SH$ group under the physical and chemical conditions which exist in living tissue is certainly autooxidizable. It is equally certain that once the $-SH$ group has given place to the $-S-S-$

FIG. 1. Curves showing uptake of oxygen by 0.5 gm. of thermostable muscle residue (rabbit) in phosphate buffer solution at pH 7.6 and 20°C. (a) In the presence of 16 mg. of glutathione (upper curve); (b) in the presence of 4 mg. of glutathione (middle curve); and (c) by tissue residue alone without dipeptide (lower curve). The glutathione was added in the oxidized form which absorbs no oxygen until reduced by the tissue.

It is seen that a 4-fold increase in the concentration of the dipeptide, while increasing the initial velocity of uptake, had no effect upon the total amount of oxygen absorbed. The curves indicate that the extracted residue contains a definite limited amount of material readily oxidizable in the presence of glutathione, but oxidized only very slowly in its absence.

group as the result of such oxidation there are factors in the tissues which promptly restore equilibrium by fresh reduction of the latter. This ability to reduce the sulfur groupings is displayed in high degree by a tissue during the first stages of its survival processes; that is, immediately after its removal from the body, though the intact tissue loses it in process of time. It follows that when the —SH group is undergoing oxidation, or (in a possible case) when its hydrogen is being transferred to some other acceptor, a certain concentration of this oxidizable group is maintained so long as the tissue contains transponible hydrogen for the purpose. Whether, therefore, the quantitative importance of this process in the tissues be small or large it seems to follow from the properties of the sulfur group and from the particular oxidation and reduction potentials which exist in the tissues that glutathione *must* play a part in certain respiratory activities. What is remarkable, however, is the fact that its part in the tissues should apparently be played, not in connection with processes of a familiar type; not, that is to say, with chemical reactions controlled by enzymes or unstable catalysts, but in relation with tissue elements which are heat-stable and, directly, at least, with materials which are insoluble in aqueous media. It should be understood that although little has been said of the matter in the present communication there is evidence to show that soluble metabolites do, when proper conditions are secured, come under the influence of what we have called the thermostable system. The facts which bear upon this statement will be given in a later paper.

It is scarcely possible to deny that the thermostable residue described in this paper as reducing and "respiring" in the presence of the sulfur group of glutathione (but effectively, only in its presence) really represents—doubtless imperfectly—a system with actual functions in the living cell.

A freshly excised normal tissue freely reduces the disulfide group in neutral solution and at the temperature of the body (a property which chemically speaking, is itself exceptional); the same tissue after thorough washing with water or normal saline solution exhibits still the same property, and no one probably will deny that this is due to factors surviving from the original tissue. When, however, the washed tissue residue is subsequently repeatedly extracted with hot water the final residue retains

almost undiminished the reducing power in question. Whatever factors are then responsible it is scarcely logical to suppose that essentially they are other than those which confer the same property on the original tissue. Yet it is precisely this power of reducing the disulfide group to the autooxidizable sulfydryl group that makes any system containing glutathione an active mechanism.

It is noteworthy that the activities which are established when glutathione is brought into relation with a tissue which has been simply well washed with cold water show little if any quantitative difference if the same tissue has been first completely extracted with boiling water. They are scarcely affected if the tissue be finally dried and powdered. In this particular relation the properties of what we have called the thermostable residue are identical with those of a tissue simply washed.

It is certainly not easy to picture what precisely can be the factors of constitution in the thermostable residue which determine its peculiar relations with sulfur groupings. It is sure that it does not act exclusively in a physical sense as a colloidal system. The fact that the uptake of oxygen, which it exhibits in the presence of glutathione is associated with a definite output of CO_2 shows that materials are actually oxidized in the system. The dipeptide in conferring activity upon it undergoes itself no change other than reversible changes which are confined to the sulfur group. The materials oxidized are, therefore, certainly present in the tissue residue itself, and are apparently very closely associated with the remains of structural elements. They do not represent merely a residuum from soluble metabolites imperfectly removed during the treatment. When a tissue has been well washed and then twice or thrice extracted with boiling water no amount of subsequent extraction affects the extent of oxygen uptake which occurs when the preparation is afterwards aerated in the presence of glutathione. In the case of muscle this uptake seems indeed to have a definite and characteristic value, being nearly the same in preparations made from the muscle of different individuals, and, to judge from a few observations, even of different species.

The nature of the materials thus closely associated with the solid elements of a tissue, and displaying towards oxygen the peculiar relations described, is as yet obscure. They are not apparently

of a lipoid nature because extraction of a dried preparation with
alcohol and ether does not diminish its activity.   It is remarkable
that during the course of oxygen uptake the respiratory quotient
of the thermostable system characteristically falls from a high
initial value to one which is near zero.

It is clear that in the oxidations (anaerobic or aerobic) involved
the dipeptide accelerates transport and acts as a "carrier."   In
this sense it is a catalyst.   It would seem that catalytic functions
of a kind must also be ascribed to the residual tissue structures in
spite of the fact that their influence withstands the effects of heat.
We have at least been quite unable to find any substance physio-
logical or other which by itself in neutral aqueous solution and at
ordinary temperatures is capable of reducing the disulfide group,
or upon the oxidation of which under such circumstances the
dipeptide exerts any influence.   It is possible that the molecules
which suffer oxidation are in some way orientated as the result
of their association with tissue structures.   If so, the configura-
tion must be very stable.

While there is much that is obscure in the phenomena involved
the facts in our opinion fully justify the claim that a non-enzymic
oxidation-reduction system represented by the thermostable
residue *plus* the sulfur grouping of glutathione actually functions
in the cell.

It is, of course, quite another matter to decide what actual share
of the normal respiratory activities of a living tissue can be
attributed to the chemical mechanism under consideration.   It
is, indeed, scarcely possible to obtain real information of any such
kind from experiments such as ours, or from those of others using
a similar technique.   When the tissues are chemically dissected
and partially reconstituted, the quantitative relationships among
interacting factors and no less their spatial relations are neces-
sarily greatly disturbed.

Such technique can satisfactorily and conclusively demonstrate
the existence of a particular chemical mechanism in a tissue;
quite other evidence must decide what precise quantitative part
it plays in support of normal tissue activities as a whole.

Apart from reference to tissues normally metabolizing it is not
even a simple matter to decide what proportion of the whole
oxidation-reduction capacity possessed by a freshly excised tissue

is reproduced when glutathione alone is added to a residue which has been rendered inactive by washing.

It should be fully understood that in our experiments the reduction velocities recorded, and no less the rates of oxygen uptake, were for the most part obtained by supplying to the system constituted by a washed, or by a washed and heated, tissue suspended in buffer solution a quantity of glutathione much greater than what would be contained in a corresponding weight of the original tissue. We have usually supplied from 2 to 8 mg. per gm. of tissue, whereas 1 gm. of fresh muscle contains probably no more than 0.25 mg. It is difficult to determine what may be the potentiality of this lower concentration when it exists in proper relations with the tissue structure.

As the result for instance of heating fresh intact muscle to 100°C. the velocity with which it reduces methylene blue is very greatly reduced; so much so that a superficial observation might decide that the reducing power had disappeared, though this is by no means the case. The reduction proceeds very slowly to an equilibrium which may be reached very rapidly when sufficient glutathione is supplied. In the latter case the relative importance of the thermostable system (compared with that of other reducing systems present in the original tissue but destroyed by heat) may seem to be exaggerated by an artificial increase in one of its constituent factors. It is equally sure on the other hand, that relying on the velocity of change, its importance would be much underestimated if the behavior of the heated fresh tissue without addition were alone considered. It would be entirely wrong to assume from this that with the relatively low concentration of glutathione present in fresh muscle the reducing mechanism described in this paper is under normal circumstances of quite small quantitative importance. A cell constituent possessing functions such as those claimed for glutathione can scarcely fail to have in the intact tissue an unequal distribution. It will be locally concentrated ·in correlation with its functions. The disintegration due to such treatment as heating to 100°C. must, especially in the case of a very soluble and diffusible substance, gravely disturb such efficient distribution. Moreover, in experiments upon methylene blue reduction, or upon oxygen uptake, the tissue must almost necessarily be suspended in a fluid,

and into this soluble constituents diffuse. The effective local concentration becomes, therefore, greatly reduced. If it is to be restored by increasing the concentration in the fluid this increase may need to be considerable.

In our experiments with preparations deprived altogether of their stock of glutathione by extraction with water, cold or hot, the dipeptide was restored by adding it to the fluid in which they were suspended to the extent of about 1 to 2 parts per 1,000 (about 0.002 M). Although as already stated the artificial system (tissue preparation *plus* fluid) contained, as a whole, much more of the substance than would appertain to a corresponding weight of the original tissue, it is unlikely that the concentration maintained at the actual locus of change was above normal. It was quite probably much less. The reduction, it should be understood, proceeds within, or at the surface of, a solid phase (the tissue residue), and not in solution.

For any attempted appraisement of the relative quantitative importance of the phenomena involved, comparisons of final equilibria are, owing to limitations in the available experimental methods, perhaps more significant than comparisons of velocity.

Even from these, however, it is difficult to draw satisfactory conclusions. A dry preparation from muscle, representing what we have called throughout this discussion the thermostable residue when "respiring" under the influence of glutathione, takes up in all about 400 c.mm. of oxygen per gm. This amount appears to be characteristic and constant for preparations made on the lines described in the last section. Calculated for moist tissue it means an uptake of rather more than 100 c.mm. per gm. If we take Thunberg's figures for the respiration of excised frog muscle at 20°C. this corresponds with the consumption of the intact tissue for a period of $1\frac{1}{2}$ hours. If, again, we take Verzar's figure for the respiration of mammalian muscle *in situ* the above figure corresponds to the consumption of something less than $\frac{1}{2}$ hour. It is not easy to determine satisfactorily what is the total capacity for oxygen uptake possessed by a given weight of fresh excised muscle. To judge from approximate data obtained by ourselves the uptake of the thermostable system is about one-tenth of the total possible uptake of the tissue from which it is prepared.

Such figures, however, have but a limited application. They give no conclusive information as to the relative importance of events in a tissue normally metabolizing. What is sure (as it seems to us) is that in the living cell catalysis by enzymes is associated with quite another type of catalysis. How they are related in the chemical organization of the cell as a whole can only be established by much further study.

SUMMARY.

When a tissue is washed until it has lost its power of reducing methylene blue the subsequent addition of glutathione to a buffer solution in which the tissue residue is suspended restores reducing power.

This is the case when the dipeptide is added in its oxidized (disulfide) form. The tissue residue first reduces the sulfur group and a system is thus established which under anaerobic conditions continuously reduces methylene blue until an equilibrium is reached.

A tissue washed until it no longer "respires" will, when suitably treated and supplied with glutathione, again take up oxygen and yield carbon dioxide.

Such part of its reducing power and respiratory activity as is regained by a washed tissue on the restoration of glutathione remains almost unaffected when the tissue is heated to 100°C. or even thoroughly extracted with boiling water.

The residue from muscular tissue so heated and extracted will in the presence of glutathione take up about 400 c.mm. of oxygen per gm. of dry material. During the earlier stages of oxygen uptake the quotient $\frac{CO_2}{O_2}$ is usually about unity; later it falls to lower values.

Glutathione does not appear to be a coagent in any known enzymic system.

The facts suggest that coexisting in living tissues with the specialized enzymic mechanisms is a thermostable mechanism for oxidations and reductions. Materials in some close association with structural elements are oxidized, anaerobically or aerobically, with the coagency of the sulfur group in glutathione.

BIBLIOGRAPHY.

Batelli, F., and Stern, L., See Stern, L., "Ueber den Mechanismus der Oxydationsvorgänge im Tierorganismus, Jena, 1914.
Hopkins, F. G., *Biochem. J.*, 1921, xv, 286.
Meyerhof, O., *Arch. ges. Physiol.*, 1918, clxx, 428.
Morgan, E. F., Stewart, C. P., and Hopkins, F. G., *Proc. Roy. Soc. London, Series B*, 1922, xciv, in press.
Thunberg, T., *Skand. Arch. Physiol.*, 1920, xl, 1.
Wieland, H., *Ber. chem. Ges.*, 1914, xlvii, 2085.

NOTE ON A COLORIMETRIC METHOD FOR THE DETER-MINATION OF SMALL AMOUNTS OF MAGNESIUM.

By F. S. HAMMETT and E. T. ADAMS.

(*From The Wistar Institute of Anatomy and Biology, Philadelphia.*)

The recent publication by both Briggs[1] and Denis[2] of colorimetric methods for the determination of small amounts of magnesium based on the colorimetric procedure for phosphorus of Bell and Doisy,[3] led us to compare our method of separation of the NH_4MgPO_4 precipitate by filtration[4] with their method of separation by centrifugation.

It was found that separation by filtration and subsequent digestion of the asbestos and precipitate with dilute HCl invariably gave results which were slightly greater than those obtained by centrifuging off the NH_4MgPO_4 and solution of the dried, washed precipitate in the centrifuge tube. A search for the source of this discrepancy led to the discovery that the best acid-washed asbestos contains sufficient phosphorus, or other substances capable of giving the Bell-Doisy color reaction, to yield a well defined colorimetric test. A similar finding was obtained with pulp made from the best grade of filter paper.

It is therefore obvious that the separation of NH_4MgPO_4 by filtration is attended with the possibility of a difficultly controllable plus error, and that separation by centrifugation as proposed by both Briggs and Denis is to be preferred.

We have, therefore, modified our procedure, in that we now precipitate the magnesium as previously described in 25 cc. centrifuge tubes, *scratch the sides of the tubes well*, and allow the mixture to stand over night. The precipitate is centrifuged off,

[1] Briggs, A. P., *J. Biol. Chem.*, 1922, lii, 349.
[2] Denis, W., *J. Biol. Chem.*, 1922, lii, 411.
[3] Bell, R. D., and Doisy, E. A., *J. Biol. Chem.*, 1920, xliv, 55.
[4] Hammett, F. S., and Adams, E. T., *J. Biol. Chem.*, 1922, lii, 211.

washed twice with 10 per cent NH_4OH and once with ammoniacal alcohol, dried, and dissolved in 10 cc. of 0.1 N HCl in the tube. The subsequent procedure is as already described.

We have also found that a cause of the fading of the color produced in the Bell-Doisy method, mentioned by them, by Randles and Knudson,[5] and by Briggs[1] is the use of old carbonate-sulfite solutions in which much oxidation of sulfite to sulfate has occurred. Possibly intermediate products of the reaction (catalyzers of oxidation) are contributing factors. That the color fading is due to oxidation, is shown by the fact that it begins at the surface of the liquid in the neck of the flask. We, therefore, use carbonate-sulfite solutions not older than 2 weeks and which have been kept in tightly stoppered 500 cc. bottles.

Differences in color due to unequal oxidation are done away with by pouring a few cubic centimeters from the second test solution at the same time the colorimeter cup is being rinsed with the first. In the magnesium determinations, however, one standard is used for each test solution because of the small final volume.

[5] Randles, F. S., and Knudson, A., *J. Biol. Chem.*, 1922, liii, 53.

METABOLIC DISTURBANCES IN CATS ON A MILK DIET.*

By GEORGE W. PUCHER and KARL F. CORI.†

(*From the Department of Laboratories of the Buffalo General Hospital and the State Institute for the Study of Malignant Disease, Buffalo.*)

The cats used in our studies on the effect of x-rays on metabolism were maintained on a milk and meat diet. This being a common food for domesticated cats, should produce no metabolic disturbances. However, these animals behaved contrary to all our expectations. The urine, instead of being acid, was intensely alkaline to brilliant yellow paper, and possessed strong reducing properties (Benedict's solution). Furthermore, the odor of the urine became very disagreeable and the animals suffered from intermittent diarrhea. This production of diarrhea in cats after the ingestion of milk was first observed by Magnus[1] and utilized by him to study the action of morphine. That these phenomena were not accidental is shown by the fact that when water was substituted for milk, a strongly acid urine, normal for carnivorous animals, was excreted. As soon as milk was again introduced into the diet, the same symptoms as previously mentioned were developed. Such profound alterations in metabolism on the mere addition of milk is not only very startling, but may be of value in understanding those diseases of babies attributed to cows milk. With this viewpoint in mind the following preliminary experiments were undertaken to determine the salient factors involved in this curious phenomenon.

EXPERIMENTAL PART.

Qualitative Observations.— Five ordinary cats, fully grown, varying in weight from 4 to 6 kilos were fed once a day with 150 gm. of meat and 100 to 150 cc. of water. The urine so collected, preserved from bacterial action with toluene, was light yellow or

---

[1] Magnus, R., *Arch. ges. Physiol.*, 1906, cv, 320; 1908, cxxii, 210.

brown in color, practically odorless, and contained only a small quantity of sediment. It was acid to litmus, brilliant yellow, and phenolphthalein. It did not reduce Benedict's solution (5 minutes boiling), and contained only a very small amount of carbon dioxide. No differences were observed between male and female animals. When 150 cc. of milk in addition to the meat were fed, within the first 24 hours, the urine of all five cats became a dirty gray in color, very turbid, and contained considerable sediment most of which consisted of triple phosphates. Only a very few scattered epithelial cells and no pus cells were present; no positive test for albumin could be obtained. These observations indicate that cystitis may be excluded. The odor of the urine became very intense and the reaction strongly alkaline to brilliant yellow, while in some instances even alkaline to phenolphthalein. All samples reduced Benedict's solution (5 minutes), and contained large amounts of bicarbonates. Diarrhea always developed in the first 24 hour periods and continued intermittently over the whole course of the experiments. The animals, however, did not appear sick and lost no weight. The milk was taken greedily by the animals, and was apparently thoroughly enjoyed. As a matter of fact, the cats preferred the milk to the meat and generally consumed this part of the diet first. The same type of reactions were obtained, *over the whole experimental period* although the intensity varied from day to day. At no time, even after a period of 3 weeks, was the urine acid to brilliant paper or free from large quantities of bicarbonates. When the milk was withdrawn and water substituted, the animals always gave the normal urine previously described.

These experiments show that the administered milk was the cause of this unique change in metabolism and that it was not a peculiarity of one particular cat but a general phenomenon of a variety of cats picked up at random.

Quantitative Experiments.—The cats were kept in ordinary wire cages with inclined galvanized iron bottoms fitted with drainage pipes connected to bottles containing about 10 to 15 cc. of toluene. The animals were fed once a day at 10.30 a.m. The cups containing the milk or water were fastened to the cages in such a way that spilling of the contents was excluded. Well nourished, full grown, healthy cats, which had been adapted to cage life were employed in these experiments. The cats showed no signs of

excitement or restlessness and were exceptionally good animals for metabolism experiments.

The food used consisted of fresh meat without bones or fat and of pasteurized cows milk obtained from the hospital kitchen. The food was always completely consumed. For this preliminary study the following substances were determined.

Total Nitrogen.—Folin's microchemical Kjeldahl method was used.

Ammonia.—Folin's and McCallum's procedure was employed.

Sugar.—The urine was first tested qualitatively with Benedict's solution and if positive run quantitatively as in the Myers and Benedict blood sugar method. It had been determined by experiment that in the dilutions used (2 cc. of a 1:10 dilution) the color produced by the creatinine could be neglected.

Total Carbon Dioxide.—This was determined, in the Van Slyke apparatus, on the urine after saturation of a sample with CO_2 at alveolar air tension. Although this procedure does not give the actual amount of carbon dioxide present at the time the urine leaves the bladder, nevertheless it is the only way consistent results could be obtained. By this method the same values for the carbon dioxide content were obtained whether the sample was analyzed immediately after voiding or 24 hours thereafter. These values are probably very near the actual ones, since Gamble[2] has shown that the elimination of carbonic acid in the urine is determined by the carbon dioxide tension of the blood plasma. For the sake of convenience the total carbon dioxide has been calculated over to sodium bicarbonate and referred to as such in the discussion. Of course, in those instances in which the urine is acid to phenolphthalein there is no doubt that the carbon dioxide evolved represents the bicarbonate that is present. However, in those experiments in which the urine was also alkaline to phenolphthalein a large part of the carbon dioxide is in the form of carbonates, but from the data at present available the carbonate-bicarbonate ratio cannot yet be evaluated.

Acidity.—The degree of acidity or alkalinity is expressed in terms of cc. of 0.1 N alkali or acid, using phenolphthalein as indicator.

The data obtained by these procedures are presented in Tables I and II.

[2] Gamble, J. L., *J. Biol. Chem.*, 1922, li, 295.

Weight of ♀ cat = 4.5 kilos. Weight of ♂ cat = 3.7 kilos.

Date.	Time inter- val.	Volume.		Specific gravity.		Sugar.		Acidity. + = 0.1 N NaOH − = 0.1 N H₂SO₄		NaHCO₃ (calculated from total CO₂).		Ammonia (NH₃).	
		♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	
1922	hrs.	cc.	cc.			gm.	gm.	cc.	cc.	gm.	gm.	gm.	g
Apr. 30	48	170	180			0	0	+58.0	+46.0			0.56	0.
May 1	48	200	210			0	0	+18.0	+46.0			0.45	0.
" 3	24	120	90	1.052	1.052	0	0	+64.0	+43.0	0.02	0.02		
" 4	24	80	125	1.056	1.046	0	0	+48.0	+63.0	0.02	0.02		
" 5	24	110	140	1.060	1.042	0	0	+58.0	+66.0	0.02	0.02	0.42	0
" 6	24	170	165	1.036	1.030	2.35	2.28	+18.0	+61.0	0.58	0.48	0.58	0
" 7	24	70	155	1.048	1.036	0.94	1.78	− 5.6	− 6.9	0.45	0.86	0.35	0
" 8	24	85	195	1.050	1.050	1.5	3.9	+15.3	+37.0	0.20	0.54	0.33	0
" 9	24	95	0	1.050		0.85		+33.0		0.04		0.34	
" 10	24	0	125		1.046	2.09			+17.9		0.30		0
" 11	24	95	230	1.048	1.044	0.95	3.48	+19.8	+ 9.0	0.078	0.953	0.43	0
" 12	24	120	155	1.042	1.038	1.52	2.48	+15.6	+24.8	0.19	0.46	0.42	0

itrogen.	$\dfrac{NH_3}{\text{Total nitrogen}}$		Diet.	Remarks.
♂	♀	♂		
$gm.$				
6.5	0.071	0.037	150 gm. meat + H_2O.	⎫ Normal period.
5.0	0.074	0.052	150 " " + "	⎪
			150 " " + "	⎬ Meat was thrice extracted
			150 " " + "	⎪ with hot water before feed-
	0.066	0.051	150 " " + "	⎭ ing.
3.8	0.12	0.14	150 gm. meat + 150 cc. milk.	Trace of diarrhea.
4.8	0.14	0.12	150 " " + 150 " "	Diarrhea.
	0.11		150 " " + 150 " "	Urine dirty gray in color.
	0.076		150 " " + 150 " " (?)	Odor very bad.
5.1		0.11	150 " " + 150 " "	
8.2(?)	0.13	0.12	150 " " + 150 " "	
4.5		0.10	150 " " + 150 " "	

Weight of ♀ cat = 4.5 kilos.   Weight of ♂ cat = 3.7 kilos.   TAƁ

Date.	Time inter-val.	Volume.		Specific gravity.		Sugar.		Acidity. $+ = 0.1$ N NaOH $- = 0.1$ N H$_2$SO$_4$		NaHCO$_3$		Ammoni (NH$_3$).	
		♀ (3)	♂ (4)	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂
													Sect
1922	*hrs.*	*cc.*	*cc.*			*gm.*	*gm.*	*cc.*	*cc.*	*gm.*	*gm.*	*gm.*	*g*
May 27	24	160	175	1.058	1.050	0.0	0.0	+ 74	+101	0.02	0.02	0.50	0.
" 29	48	175	175	1.062	1.050	0.0	0.0	+ 93	+ 87	0.04	0.04	0.50	0.
" 31	48	135	205	1.060	1.050	0.0	0.0	+ 72	+123	0.03	0.04	0.46	0
June 2	48	150	225	1.062	1.048	0.0	0.0	+ 71	+133	0.032	0.045	0.35	0
" 3	24	80	105	1.060		0.0	0.0	+ 50	+ 46	0.02	0.02	0.20	0
													Sec
June 4	24	147	133	1.050	1.050	1.5	1.3	+2.9	+ 42	0.47	0.06	0.42	0
" 5	24	275	325	1.022	1.022	2.0	3.6	−384	− 72	7.4	2.1	2.5	1
" 6	24	135	200	1.040	1.030	0.73	2.2	−146	+ 34	1.8	1.2	0.93	0
" 7	24	105	140	1.040	1.030	0.71	1.2	− 53	− 22	0.93	0.60	0.52	0
" 16	24	160	165	1.030	1.034	++	+	−155	+ 19	1.41	0.88	1.06	0
" 17	24	197	165	1.036		1.55	2.47	−276	+ 29	4.05	1.36	1.63	
													Se
June 20	48	446	400	1.034	1.020	6.7	10.0	−772	+ 84	11.1	4.4	4.08	
" 22	48	400	450	1.026	1.028	+	+++	Alka-line.	Alka-line.				
" 23	24	330	425	1.028	1.040	4.6	14.0	−198	− 85	4.9	2.3	2.08	
" 24	24	178	415	1.030	1.026	0.71	5.4	− 71	− 26	1.7	1.8	1.22	
" 26	24	225	327	1.028	1.030	0.0	6.5	−162	− 33	3.2	1.4	1.9	
													S
June 28	48	150	275	1.014	1.030	0.90	5.2	− 15	+ 66	1.57	1.63	0.95	
" 29	24	123	177	1.022	1.024	1.04	2.5	−102	+ 14	1.8	0.44	0.88	
July 1	48	250	290	1.024	1.030	1.9	5.2	−115	−110	2.7	3.6	1.4	
" 3	48	242	340	1.028	1.030	2.1	4.4	−162	−173	3.8	3.8	1.6	
" 4	24	98	100	1.040	1.036	0.0	0.0	+21.6	+ 40	0.48	0.48	2.4 (?)	
" 5	24	50	105	1.060	1.046	0.0	0.0	+28	+ 56	0.05	0.03	0.57	
" 6	24	75	80	1.054	1.040	0.0	0.0	+53	+ 40	0.03	0.02	0.20	

al gen.	NH₃/Total nitrogen		Diet.	Remarks.
♂	♀	♂		
gm.				
	0.093		150 gm. raw meat + H₂O.	
9.7	0.062	0.034	150 " " " + "	
13.0	0.062	0.041	150 " " " + "	Normal period.
13.5	0.048	0.051	150 " " " + "	
6.0	0.063	0.031	150 " " " + "	
5.0	0.107	0.05	150 gm. raw meat + 200 cc. milk.	
4.7	0.80	0.25	150 " " " + 200 " "	Strong diarrhea.
4.5	0.29	0.17	150 " " " + 200 " "	Faint diarrhea.
3.0	0.10	0.12	150 " " " + 200 " "	During this week no quantitative data. Urine always alkaline and reduced Benedict's solution.
3.5	0.29	0.16	150 " " " + 200 " "	Sediment chiefly triple phosphates. No albumin.
3.5	0.38	0.21	150 " " " + 200 " "	Strong diarrhea.
3.5	1.02	0.47	300 cc. milk.	Diarrhea. Only a few crystals of triple phosphates in sediment.
			300 " "	
2.7	0.65	0.49	300 " "	
1.1	0.94	1.08	300 " "	
1.2	1.12	0.96	300 " "	
3.9	0.40	0.29	150 cc. milk	
2.1	0.63	0.20	150 " "	
3.2	0.46	0.53	150 " "	Strong diarrhea.
2.9	0.73	0.69	150 " "	Very flocculent yellow sediment. Diarrhea.
3.1	0.67	0.17	150 gm. meat + H₂O.	
4.4	0.21	0.038	150 " " + "	Urine clear as in Section A.
3.2	0.067	0.041	150 " " + "	

DISCUSSION OF TABLES.

Table I.—It will be noted that within 24 hours after the feeding of the milk, the urine contains nine times as much bicarbonate as during the normal period. This large excretion of bicarbonate is not constant, but always markedly higher than in the normal period. It will also be noted that the total acidity is decreased as the bicarbonate content increases. The ammonia-total nitrogen ratio is also increased 1.5 times under the influence of the milk diet. All urines on a milk diet showed a positive Benedict's test and varied in their total sugar excretion as shown in Columns 7 and 8. (See discussion concerning this reducing substance.) It will be observed that the male cat always has a higher excretion of sugar than the female.

Table II, Section A.—This simply represents a normal period on a meat and water diet.

Table II, Section B.—The addition of 50 cc. more of milk to the diet increased the symptoms recorded in Table I to a much greater degree. Within 48 hours after the addition of the milk the bicarbonates increased in the case of the female cat 350 times over the normal value, while in the male cat, it was 100 times over the normal. It will be noted that the urine in both animals, with the increased elimination of bicarbonates, has become strongly alkaline even to phenolphthalein. The ammonia-total nitrogen ratio increased in the female cat ten times and in the male cat six times over that of the normal period. The reducing properties of the urine on this diet was about the same as in Table I.

Table II, Section C.—These experiments are a continuation of those in Section A. The meat diet was eliminated and the milk increased to 300 cc. The animals lost no weight during this 5 day period and the symptoms were of the same general character as recorded in the other sections. However, it will be noted that the ammonia-total nitrogen ratio as well as the amount of bicarbonate excreted was higher than in any of the other protocols.

Table II, Section D.—This is a continuation of Section B in which the milk was cut down to 150 cc. This was practically a starvation diet. The results are similar to those reported in the preceding sections, the urine continuing to be alkaline and the ammonia-total nitrogen ratio far above its normal value. To

see how long a period was required to bring the animals to their normal state, (latter half of Section D), after a 3 weeks milk diet the normal diet (150 gm. of meat and water) was again fed. In 24 hours the urine of both animals was acid to phenolphthalein and contained no reducing substances. The bicarbonate and ammonia-total nitrogen ratio were still high but within 48 hours this had also returned, in the case of the male cat, to its normal value. The female cat required 96 hours to reach the values of the normal diet.

DISCUSSION OF DATA.

Our qualitative as well as quantitative results prove that cats suffer a disturbance in their alkali metabolism when on a milk

TABLE III.

Summary of Data.

	Normal period. Meat and water. Average amount excreted in 24 hrs.		Milk diet. Average amount excreted in 24 hrs.	
	♀	♂	♀	♂
Volume, cc....................	91.0	109.0	148.0	187.0
Specific gravity.................	1.059	1.048	1.034	1.030
Sugar, gm.....................	0.0	0.0	2.7	7.5
Acidity,* cc....................	+41.0	+50.0	−108.0	− 5.2
$NaHCO_3$, gm.................	0.017	0.018	1.94	1.16
NH_3, gm.	0.288	0.245	1.01	0.79
$\dfrac{NH_3}{\text{Total nitrogen}}$	0.065	0.049	0.405	0.282

* + = $0.1 N$ NaOH.
  − = $0.1 N$ H_2SO_4.

diet. This is particularly shown by the huge amounts of bicarbonate eliminated by the animals on such a diet. Animals fed on meat and water excrete only a small, but very constant amount of bicarbonate. This observation is in harmony with that of Gamble[2] on human urine.

In order to obtain a better contrast between cats on a normal diet and those fed with milk Table III is recorded. The figures represent the average sum of the substances excreted for 24 hours on the two diets.

TABLE IV.

Weight of cat = 2.15 kilos.

Date.	Time.	Volume.	Specific gravity.	Sugar.	Acidity.	NaHCO₃	NH₃	Total nitrogen.	NH₃ / Total nitrogen	Diet.	Remarks.
1922	*hrs.*	*cc.*		*gm.*		*gm.*	*gm.*	*gm.*			
May 24	24	148	1.037	0	60.0	0.081	0.203	4.7	0.043	150 gm. meat + H₂O.	
" 26	48	198	1.05	0	120.0	0.054	0.44	7.8	0.057	150 " " +	
" 29	48	200	1.037	0	63.0	0.103	0.31	5.7	0.055	150 " " +	
" 31	48	125	1.062	0	77.0	0.016	0.44	7.6	0.058	7 gm. lactose in water.	
June 3	48	127	1.054	0	45.0	0.041	0.26	5.9	0.044	10 " "	
" 5	48	156	1.061	0	112.0	0.075	0.35	8.2	0.045	15 " "	Diarrhea.
" 7	48	130	1.065	0	92.0	0.071	0.38	7.2	0.053	20 " "	
" 9	48	144	1.060	0	74.0	0.067	0.30	6.8	0.057	20 " "	Diarrhea.
" 12	48	188	1.072	+	92.0	0.08	0.56	6.8	0.083	25 " "	
" 14	48	82	1.064	0	110.0	0.19	0.24	4.5	0.055	20 " "	
" 16	48	143	1.057	0		0.05	0.32	6.7	0.048	25 " "	Diarrhea.
" 19	48	114	1.060	+	87.0	0.054	0.36	6.8	0.053	30 " "	Diarrhea.
" 21	48	158	1.038		72.0	0.048	0.31	4.8	0.065	"	"

The mechanism of these changes in the metabolism of the cats or the substances present in the milk which induce these changes have not yet been completely determined. However, it has been proven that lactose alone is not responsible for any of the effects observed. This is demonstrated by the data in Table IV.

It will be observed that on the administration of lactose the urine in no case became alkaline (to brilliant yellow or phenolphthalein) or increased in bicarbonate content. The ammonia-total nitrogen ratio remained constant during the entire experimental period. It will also be observed that even though the animals suffered from diarrhea on an excess of lactose, none of the symptoms similar to those shown on a milk diet were developed. This indicates that the marked disturbances in cats produced by milk are probably independent of the occurrence of the diarrhea.

These experiments also indicate that cats have a relatively high lactose tolerance and that only after 25 to 30 gm. in 48 hours have been ingested is there any sugar excreted in the urine. Yet in most of our experiments in which milk was administered to the cats, a sugar reduction was obtained. It seems very improbable that this could be lactose since the amount of lactose contained in the milk was far below the determined lactose tolerance of the animals. Furthermore, no lactose could be identified (mucic acid test) in any of the urines examined. The nature of this reducing substance, for the present recorded as sugar, will be investigated later. It is also our intention to determine the constituents of the milk responsible for this change in cat metabolism and to apply, if possible, these observations to metabolic disturbances produced in babies by cows milk.

SUMMARY.

1. Cats when fed on meat and water excrete a urine normal for carnivorous animals. The total carbon dioxide content of the urine is very small and constant in value.

2. Cats when fed on milk excrete within 24 to 48 hours a urine which shows the following qualities: (a) Alkaline to brilliant yellow or even to phenolphthalein; (b) substances which easily reduce Benedict's solution (5 minutes boiling); (c) huge amounts of bicarbonates (calculated from total carbon dioxide evolved);

and (d) increase of the ammonia and the ammonia-total nitrogen ratio.

3. These symptoms were observed as long as the animals were maintained on a milk diet (3 weeks).

4. Within 48 hours after the withdrawal of the milk the animals returned to normal.

5. Milk sugar is not responsible for these changes in the alkali metabolism of the cats.

ON THE CONSTANCY OF THE CREATINE-CREATININE EXCRETION IN CHILDREN ON A HIGH PROTEIN DIET.*

By VICTOR JOHN HARDING and OLIVER HENRY GAEBLER.

(From the Department of Pathological Chemistry, University of Toronto, Toronto, Canada.)

In a general way it is usually agreed that the appearance of creatine in the urine is concomitant with a lowered creatinine excretion, and such a generalized idea has been adduced as a reason for the biochemical interrelationship of the two substances. So diverse, however, have been the conditions under which creatinuria has been observed to appear and disappear, that such a generalized expression has never been accorded the validity of a biochemical law.

The clearest example of the reciprocal relationship of creatine and creatinine, and the consequent constancy of the excretion of the sum of both in an individual, is perhaps to be found in the work of Benedict and Diefendorf (1) on starvation in a woman. Goldschmidt, Pepper, and Pearce (2) point out the constancy of the total creatinine (creatine + creatinine) in a child of $5\frac{1}{2}$ years, observed before and after splenectomy. Cameron and Gibson (3), from a study of the creatine-creatinine excretion in various cases of muscular dystrophy, and in amputations, argue strongly for the interrelationship of the two.

The clear-cut character of results such as these, however, has not always been attained, and it is such failures which have militated against the placing of the relationship of creatine and creatinine upon a quantitative basis. Thus, to cite only one recent example, we may mention the work of M. S. Rose (4) upon creatinuria in women, where after noticing that "very often when

the creatine was high the creatinine was low, and *vice versa*," she observes, "but in plotted curves the sum of the two did not make a straighter line than either one alone."

So many factors, however, have been found to influence the excretion of creatine (acidosis, dietary protein, water drinking), that such failures are not surprising, and it is only when such factors are brought under rigid control or are compensated by observations over longer periods of time and the taking of averages, that the reciprocal relationship of creatine and creatinine is shown to be quantitative.

In the present paper, we wish to show that *normal* children of the same age and under the same environment, excrete the same quantity of total creatine (creatine + creatinine in terms of creatine). And that, if the total creatine in milligrams be expressed as a coefficient of the body weight in kilos, the figure obtained is a constant, independent of the age of the child, and of the same magnitude as the creatinine coefficient of an adult man. This figure we have called the "total creatine coefficient."

The experiments upon which these conclusions are based were commenced with the object of repeating the observations of Denis and Kramer (5) upon the creatinuria of children, and we may say that our experiments are corroborative of the findings both of these workers and of Powis and Raper (6). We wish, however, in this communication, to report the results obtained on as high a protein diet as the child could conveniently consume. At the outset we wish to emphasize the normal character of our children. They were children living at an Institute[1] on the outskirts of the city, and who had been sent there as mild cases of malnutrition with a history of possible exposure to tuberculosis. In none of them, however, had there been any active infection, and all were at normal weight and actively engaged in play in the grounds of the Institution. They were thus during the experiment, and for some time previous had been, under the same environmental conditions of diet and exercise. Twelve children were selected and divided

into three groups according to age, and each group consisted of two children of each sex. They were fed at a separate table, and the diet and urine collections were made the special care of one nurse. Each group was studied separately. The diet consisted of cereals, eggs, bread, butter, milk,[2] potato, vegetables, sugar, and oranges. The amount taken at each meal was charted, and the nitrogen intake calculated. Except that the children were deprived of meat and soups made from meat stock, the diet did not differ markedly from that to which they were accustomed, and as meat was in the ordinary routine, only supplied once a day, the experimental period represented no sharp break from their customary diet. In Table I are presented the average figures for a 3 day period for each child when on the high protein diet. The constancy of the nitrogen excretion of each group upon the same diet will be noted.

Group A, the youngest children, shows a remarkable constancy in the total creatine excretion, each child varying but a few milligrams from the average of the whole group (344 mg.). Group B also shows a good agreement in the excretion of total creatine by each child, and it is Group C which shows the greatest discrepancies. In this last group, it would almost appear that the boys were separating from the girls. If, however, we compare the total creatine coefficients with the creatinine coefficients, the reciprocal nature of creatine and creatinine in children comes clearly to light. The creatinine coefficients range from 10.1 to 18.2, whereas the total creatine coefficients vary from 21.3 to 27.6, and this last figure stands alone in this series, a much better high limit being 24.7. The averages for each group too are instructive. The average creatinine coefficient for each group rises definitely with increasing age and weight, accompanied as it is by an increasing percentage of musculature. The total creatine coefficient, however, is remarkably steady around the total average of 23.1. Particularly interesting too, is the case of L. G. in Group C. This girl was a big, heavy girl, overweight according to her age, and whose creatinine coefficient was distinctly under the average of her group. Yet, she excreted nearly half as much creatine again,

[2] The ingested creatine coming from the milk amounted to 20 to 30 mg. per day. This amount is of insignificance compared with the total excretion.

as her companion girl in the same group, and her total creatine coefficient becomes almost exactly the same as the total average.

These findings, like those of Folin and Denis (7) and Denis and Kramer (5), receive their simplest interpretation by assuming that

TABLE I.*

Child.	Sex.	Weight.	Nitrogen intake.	Total urine nitrogen.	Average creatinine.	Creatinine coefficient.	Average creatine excretion.	Total creatine.	Total creatine coefficient.
Group A. Ages 2½ to 3½ yrs.									
		kg.	gm.	gm.	mg.		mg.	mg.	
M.W.	F.	14.0	12.1	6.13	173	12.4	138	338.68	24.19
H.C.	"	14.0	12.1	6.78	141	10.1	145	308.56	22.04
G.L.	M.	14.5	12.1	6.21	180	12.5	121	329.80	22.74
V.D.	"	15.0	12.1	6.85	200	13.6	125	357.00	23.80
Group average						12.1			23.2
Group B. Ages 4 to 4½ yrs.									
A.M.C.	F.	16.5	11.4	6.25	223	13.7	100	358.68	21.7
D.F.	"	19.1	11.4	7.34	276	14.2	109	429.16	22.4
W.R.	M.	19.0	11.4	8.43	281	15.0	90	415.96	21.9
A.L.	"	17.0	11.4	7.00	261	15.6	77	379.76	22.4
Group average...........................						14.6			22.1
Group C. Ages 9 to 9½ yrs.									
C.A.	F.	28.8	15.6	10.94	524	18.2	187	794.84	27.6
L.G.	"	33.5	15.6	10.06	439	14.1†	259	769.24	22.9
R.A.	M.	25.0	15.6	10.61	454	18.0	90	616.64	24.7
S.G.	"	27.6	14.3	9.13	476	17.6	36	588.16	21.3
Group average...........................						18.1			23.9

*Analytical Methods.—Total nitrogen, micro method of Gulick. Creatine and creatinine, micro method of Folin using creatinine-zinc chloride as standard. pH, method of Palmer and Henderson; these never showed any significant variations.

† Excluded from average.

creatine is produced from protein either *in toto* or from a special fraction of it, is stored in the muscles, and is converted into creatinine. With the saturation of the muscles with creatine, any excess production will find its way into the urine, and provided the

dietary protein is not reduced to such a low level that a condition of protein starvation occurs, the urinary creatine will appear as a waste product of exogenous origin. If, however, the constancy of the total creatine coefficient, when upon a high protein diet, is admitted, it would involve the conclusion that creatine production reaches a maximum, and that once that maximum is reached no amount of further feeding of protein, however excessive, will result in an augmented creatinuria. Similarly, moderate decreases in protein intake may fail to lower or abolish an existing creatinuria. That such a maximum probably exists can be shown experimentally in dogs, and these results will be reported later.

The acceptance of these conclusions also involves a further corollary that the muscular system does not control the production of creatine. It is certainly remarkable that a child of 3 years should produce per kilo body weight as much total creatine as an adult man. The muscular system of the child has not reached its maximum percentage of the total body weight, and the relative creatinine production is low. It would thus appear that creatine disappears from the urine of the adult man when the creatinine coefficient has attained its average maximum value, and the muscular system has also reached its average adult percentage of the total body weight. This being so, one can only agree in part with Mellanby (8) that the production of creatine is not controlled by the muscular system, which is in harmony with its phylogenetic and otogenetic history. Cameron and Gibson (3) in a recent article, have expressed a similar conclusion from a study of the creatine-creatinine excretion in muscular dystrophies and amputations.

Influence of Age and Sex upon Creatinuria.

In view of the figures given in Table I and the conclusions which have just been expressed, it is evident that some of the current conceptions on the effect of age and sex upon the creatine excretion of children, require revision. These conceptions are based upon the work of Rose (9) and Krause (10). The former drew the conclusion that creatine was present in the urine of children up to the age of puberty; the latter that creatinuria was present in boys up to the age of 5 to 6 years, but that in girls, it continued up to the age of 10. Undoubtedly, in drawing this conclusion

584   Creatine-Creatinine Excretion

Krause was influenced by his previous observations that creatinuria could occasionally be found in normal adult women. It is very evident that boys of ages higher than 5 or 6 years excrete creatine, if sufficient protein is supplied in the diet, and judging from the amounts of creatine excreted by the two girls in Group C, that they would continue excreting creatine long after the age of 10.

The relation of the creatinine coefficient to the total creatine coefficient, however, shows that on the average creatine will disappear from the urine of both boys and girls at about the age of 16 years, if each sex develops the muscular system to the same degree. This figure has been attained by plotting the group creatinine coefficients against the group age and extrapolating the curve obtained until it reaches the figure equivalent to the average total creatine coefficient. It is interesting to note that at this age the muscular system has attained its maximum development in relation to body weight forming about 44 per cent of the total (11). In thus setting 16 years as the age at which creatinuria will disappear even upon a high protein diet, it is only intended to convey that that is an average figure. Undoubtedly, cases will be found in which creatinuria persists beyond this age. Thus, Folin and Denis (7) report the case of a normal boy of 17 still excreting creatine, and undoubtedly there must be many cases where creatine has disappeared at an age earlier than the average.

As far as our figures show, there is no distinct influence of sex in the early years. Apparently, however, there is a slight divergence in later years (Group C), but the figures are not sufficiently numerous to permit of the drawing of decided conclusions. The well known results of Tracy and Clark (12) on the low creatinine coefficients of normal women as contrasted with normal men, would however, incline us to believe that taken on the average, the majority of women would show creatinuria on a high protein diet. This, we believe, in spite of the negative results of Rose (4) and Rose, Dimmitt, and Bartlett (13). In this connection, we think that an examination of the results shown by the two girls of Group C is of interest. Both girls, of the same age and upon the same diet excrete almost the same amount of nitrogen. L. G. has a low creatinine coefficient and is a fat, heavy girl. It might therefore be expected that she would continue to excrete creatine for at any rate a longer period of her life

than her companion C. A., especially as her excretion under identical conditions is nearly 50 per cent higher. Such a prediction should be true for the average case. Girl C. A., however, possesses an exceptionally high total creatine coefficient and would need to develop an exceptionally vigorous muscular system in order to attain a creatinine coefficient equivalent to 27.6. While we believe that *on the average, the height of the creatinine coefficient gives a method of predicting the presence or absence of a creatinuria upon a high protein diet we have no means of foretelling the exact height of the total creatine coefficient in the individual.*

Total Creatine Coefficient in Some Pathological Conditions.

In order to see whether the conclusions drawn from a study of the creatine excretion in children are applicable to creatinuria in pathological conditions when on high protein diets, we have tabulated in Table II some results of our own, and such results in the literature as we could find, and in which were reported the requisite data. It will be seen that with two exceptions, creatinuria in pathological conditions shows the same general rule as creatinuria in children. The total creatine coefficient averages 21.9, a figure very close to that given as the average for children of 22.7, and that the variations in the coefficient cover the same range as does the creatinine coefficient in normal man. This, we think is remarkably good agreement, as the conditions of diet were not uniform, and the investigators, including ourselves at the time of the observations, were quite unaware of the constancy of this figure. The two exceptions are possibly to be explained by the exceptionally heavy weight of the boy O. Sch., and that child D. R. was on so low a protein diet, that some of the creatine may be of starvation origin.

Particular interest attaches itself to the observations in the hyperthyroid cases reported by ourselves, Denis, and Denis and Minot. Owing to the experimental work on creatinuria in this condition, a great deal of attention has been centered on the thyroid, and Gross and Steenbock (14) have made the function of this gland an essential part of their theory of the origin of creatine from arginine. It would not appear from the results we have just given that the thyroid gland plays any direct rôle

TABLE II.

Calculation of Creatinine and Total Creatine Coefficients in Some Pathological Conditions.

Diagnosis.	Subject.	Sex.	Age.	Weight.	Average N intake.	Creatinine coefficients.	Total creatine coefficients.	Observer.
			yrs.	k.l.	gm.			
Hyperthyroid.	L. B.	F.	33	40.3	10.5	10.3	21.9	Harding and Gaebler.*
"	P. S.	"	45	50.7	7.9	9.3	17.3	"
"	A. S.	"	41	65.2	12.4	12.9	27.5	"
Acromegaly.	V. E.	"	29	61.2	10.1	14.0	20.1	"
Hyperthyroid.	I	"	33	55.2	8.25	12.6	22.5	Denis (15).
"	II	M.	35	58.0	13.53	21.7	24.9	"
"	III	F.	21	59.0	9.47	13.7	23.0	"
"	IV	"	46	52.3	8.76	12.2	16.6	"
"	V	"	23	54.5	9.97	12.8	18.9	"
"	III	"	32	45.0	13.97	16.1	27.0	Denis and Minot (16).
"	IV	"	21	41.0	10.91	15.2	24.0	"
"	V	"	28	51.0	12.11	10.5	21.3	"
"	VI	"	27	63.3	17.31	11.2	23.5	"
Muscular dystrophy.	O. Sch.	M.	15	90.0	9.62	2.82†	10.3†	Gibson, Martin, and Buell (17).
"	B. McD.	"	22	53.0	11.38	19.48	25.6	"
"	L. W.	"	25	63.0	9.92	19.7	25.4	"
"	J. Sch.	"	6	18.2	4.62	7.56	20.7	"
"	D. R.	"	8	21.8	3.96	13.53	38.2†	"
Average						13.8	21.9	

* The authors wish to thank Dr. W. Campbell of the Toronto General Hospital for his cooperation in these cases.

† Excluded from averages.

in the production of creatinuria. Acromegaly, muscular dystrophies, and children all show a power to produce the same amount of creatine per kilo body weight as the normal adult. Only in so far as the activities of the thyroid alter the proportion of muscular mass to total body weight, does that gland contribute a quota to the production of a creatinuria.

BIBLIOGRAPHY.

1. Benedict, F. G., and Diefendorf, A. R., *Am. J. Physiol.*, 1907, xviii, 364.
2. Goldschmidt, S., Pepper, O. H. P., and Pearce, R. M., *Arch. Int. Med.*, 1915, xvi, 437.
3. Cameron, A. T., and Gibson, A., *Canad. Med. Assn. J.*, 1922, xii, 393.
4. Rose, M. S., *J. Biol. Chem.*, 1917, xxxii, 1.
5. Denis, W., and Kramer, J. G., *J. Biol. Chem.*, 1917, xxx, 189.
6. Powis, F., and Raper, H. S., *J. Biol. Chem.*, 1916, x, 363.
7. Folin, O., and Denis, W., *J. Biol. Chem.*, 1912, xi, 253.
8. Mellanby, E., *J. Physiol.*, 1907–08, xxxvi, 447.
9. Rose, W. C., *J. Biol. Chem.*, 1911–12, x, 265.
10. Krause, R. A., *Quart. J. Exp. Physiol.*, 1914, vii, 87.
11. Voit, C., *Z. Biol.*, 1866, ii, 307.
12. Tracy, M., and Clark, E. E., *J. Biol. Chem.*, 1914, xix, 115.
13. Rose, W. C., Dimmitt, J. S., and Bartlett, H. L., *J. Biol. Chem.*, 1918, xxxiv, 601.
14. Gross, E. G., and Steenbock, H., *J. Biol. Chem.*, 1921, xlvii, 45.
15. Denis, W., *J. Biol. Chem.*, 1917, xxx, 47.
16. Denis, W., and Minot, A. S., *J. Biol. Chem.*, 1919, xxxvii, 245.
17. Gibson, R. B., Martin, F. T., and Buell, M. Van R., *Arch. Int. Med.*, 1922, xxix, 82.

SOME SOURCES OF ERROR IN THE DETERMINATION OF CHLORIDES IN BLOOD AND SIMILAR MATERIAL.*

By ISIDOR GREENWALD and JOSEPH GROSS.

(From the Harriman Research Laboratories, The Roosevelt Hospital, New York.)

In the course of work in the analysis of blood, the authors have employed a number of reagents for the removal of the proteins before proceeding to the determination of other constituents. Two of these, picric acid (1) and nitric acid (2), appeared to offer peculiar advantages in the subsequent determination of chlorides and other inorganic constituents. However, it was found that the amount of silver chloride that was obtained from volumes of filtrates corresponding to the same amounts of blood was much less when nitric acid was used as the precipitant than when picric acid was so employed. Moreover, the silver precipitate obtained with the latter was always yellow and it retained this color even after prolonged washing with hot water. The color could be removed by washing with concentrated nitric acid but the weight of the residual silver chloride was greater than that of the precipitate obtained from a nitric acid filtrate corresponding to the same amount of blood.

The yellow silver chloride precipitate was not completely soluble in ammonia but left a gelatinous precipitate, suggesting the presence of a purine. The precipitate was filtered out, washed, and suspended in dilute hydrochloric acid. After heating for 30 minutes, the mixture was filtered, the filtrate was almost neutralized and was then treated with sodium acetate, sodium bisulfite, and copper sulfate in the usual manner. The precipitate obtained was filtered out, washed with hot water, and decomposed with hydrogen sulfide. The filtrate from the copper sulfide was evaporated and treated with a little picric acid. On cooling, a lemon-

---

{* A preliminary report was read before the American Society of Biological Chemists, at New Haven, December, 1921.

yellow picrate crystallized. This was at first thought to be hypoxanthine picrate but later experiments have shown that it was probably a mixture of hypoxanthine and adenine picrates. Apparently, the yellow color of the original silver chloride precipitate was due to the presence of purine-silver picrates. These have been described by Bruhns (3) who ascribed to them the formula (Purine-H) $AgC_6H_2(NO_2)_3OH$. Attempts in this laboratory to prepare the hypoxanthine compound by adding silver nitrate to hypoxanthine picrate solutions or picric acid to a solution of hypoxanthine silver nitrate and excess silver nitrate have always yielded precipitates containing less hypoxanthine and more silver than this formula requires.

The ammoniacal filtrate from the purine-silver precipitate was acidified with nitric acid and the reprecipitated silver chloride was filtered on a Gooch crucible and weighed. The amount found was greater than that obtained from a nitric acid filtrate from a corresponding amount of blood. Evidently, nitric acid failed to extract all of the chloride from blood.

It seemed to be of interest to investigate other protein precipitants that have been recommended for use in the determination of chlorides in blood. Accordingly, samples of oxalated blood were treated as called for in the description of the methods. However, since it was desired not to have the results complicated by variations in the character of the end-points used in the several titrations, all the determinations discussed in this paper were made gravimetrically, either by weighing the silver chloride precipitated by the addition of silver nitrate (and nitric acid) to the protein-free filtrate or, where this was, for one reason or another, undesirable, by using a known excess of silver nitrate, removing the precipitate, and then determining the excess of silver in the filtrate by precipitation with hydrochloric acid. In many cases both methods were used. In almost every instance, filtrate equivalent to 10 cc. of blood was used. In a few cases, the equivalent of 5 cc. of blood was employed.

The results obtained are summarized in Table I. The first column of figures gives the values obtained by digestion of the blood with nitric acid and an excess of silver nitrate until all the organic matter had been dissolved. It was not necessary to use potassium permanganate (4). These are regarded as probably the most

TABLE I.

Comparison of Chloride Determinations in Protein-Free Filtrates Obtained by Different Methods.*

Method	Oxidation of whole blood with HNO₃	Picric acid — Myers and Short	Picric acid — Austin and Van Slyke	Picric acid — Ag precipitate washed with HNO₃	Nitric acid — Greenwald and Gross	Nitric acid — Rappleye	m-Phosphoric acid — Oppler	m-Phosphoric acid — Foster	Trichloracetic acid Smith	CuSO₄ + Ca(OH)₂ Wetmore	Methyl alcohol Richter-Quittner	Tungstic acid Roeger-Whitehorn	Mixture of picric and nitric acids
Sheep blood.													
Direct.	313.2	328.0		310.2	287.0	328.5	310.4	298.5	304.3	304.8	309.1	313.8	
Excess AgNO₃.		323.1								304.6			
Direct.	333.0		341.0	338.6			325.4	322.1	334.9	328.9			
Excess AgNO₃.	330.2		337.2	338.2						327.6			
Calf blood.													
Direct.	303.6	324.0	314.3	306.6	286.5	329.0	300.8	299.3	308.3	301.6	319.1	304.0	301.9
Excess AgNO₃.	303.3	316.3	313.0	307.0	290.0					303.5			300.6
Human blood.													
Direct	320.3	334.2		320.3	303.3	323.3			315.8			317.8	
Excess AgNO₃.	319.9	325.2		320.5	303.1								
Direct.	320.0	337.0	331.0	324.6	306.0		312.4	307.4	310.6	305.2	310.6	322.0	322.7
Excess AgNO₃.	321.3	326.7	326.0	321.4						305.0			321.8

* Mg. chlorine per 100 cc. blood.

accurate values and all others have been compared with them. The three following columns give the results by the three methods in which picric acid is used as the protein precipitant. As might be expected from what has been said about the occurrence of a purine-silver picrate, the results obtained by the technique of Myers and Short (5) are too high, the average error being 3 per cent. Even if 5 per cent nitric acid be present, as in the method of Austin and Van Slyke (6), the error is about the same. This 3 per cent error is found if the amount of excess silver in the filtrate from the blood chlorides is determined and used as a basis for the calculations. If these are based on the weight of the first silver chloride precipitate, the error is even greater because of the high molecular weight of the purine-silver picrate. If the precipitate is washed with concentrated nitric acid, the agreement is better, the maximum error being 2 per cent and the average less than 0.5 per cent.

If the proteins are precipitated with nitric acid and the chlorides are determined in the filtrate, the results are always too low by from 5 to 8 per cent. They are as much too high if the nitric acid and silver nitrate are combined in one solution as in the methods of Gazzetti (7) and of Rappleye (8). Evidently, there is something other than chloride in blood (not necessarily in plasma or serum, for which these methods were described) which precipitates silver from a nitric acid solution.

The filtrates from the m-phosphoric acid precipitation of blood yield on direct precipitation, very irregular results, which are all too high, probably because the precipitates are contaminated with protein. Calculation from the amount of excess silver in the filtrates gives values that are too low, the error being somewhat less with Oppler's method (9), in which an excess of m-phosphoric acid is avoided than with Foster's method (10).

The silver chloride precipitated in the filtrates, obtained by precipitating blood with 5 per cent trichloroacetic acid (11, 12, 13), even when alcohol was used as described by Smith, cannot be filtered, even after standing over night. It can, however, be removed with the aid of a centrifuge within a few minutes. An aliquot of the supernatant liquid is used for a determination of the excess silver. The values thus obtained are from 3 per cent too low to 1.7 per cent too high.

The use of copper sulfate and sodium and calcium hydroxides, as employed by Wetmore (14), gives results that are too low by from 0.3 to 5 per cent, the average error being about 3 per cent. The technique of Harding and Mason (15) is probably similarly at fault.

Direct gravimetric determination of the chloride in the filtrate from the precipitation of blood with methyl alcohol and magnesium sulfate, as described by Richter-Quittner (16) is impossible because the precipitate contains much foreign material. Calculations based upon determinations of the excess silver in the filtrate give irregular results, 5 or 6 per cent too high or too low.

The filtrate from the Folin-Wu tungstic acid precipitation, when treated with nitric acid and silver nitrate, gives a precipitate consisting of silver chloride and tungstic acid (17, 18). If this is filtered out and the amount of choride is calculated from a determination of the excess silver in the filtrate, the values obtained agree very well with those obtained by digestion of the whole blood with nitric acid and silver nitrate. Apparently, the Folin-Wu reagent is, of all those studied, the most suitable for the subsequent determination of chlorides, and, unless there is some difficulty with the subsequent titration, which appears not to be the case, the method described by Whitehorn (18) would seem to be the most desirable for routine determinations.

The high values obtained in the picric acid methods are due to the formation of a purine-silver picrate. But to what may the low values obtained with the other protein precipitants be ascribed?

It may be assumed that they are due to the presence, in the blood, of some organic chlorine compound which was precipitated by some of the reagents, or which decomposed to give chlor-ion in some of the procedures, but not in others. But it is a reagent of low acidity, tungstic acid, that gives values most nearly identical with those obtained by digestion of the whole blood with nitric acid and silver nitrate.

The slightly lower values obtained by Wetmore's method are probably due to the formation of a small amount of basic copper chloride.

The other protein precipitants are all acids and precipitation occurs only well on the acid side of the isoelectric points of the

blood proteins. The precipitate obtained is a compound of protein and acid. It has been more or less tacitly assumed that, in these reactions, the precipitating acid completely displaces all other acids from combination with the protein. But this seems not to be the case with nitric and m-phosphoric acids, and probably not with trichloroacetic acid.

It was at first thought that the amount of chlorine retained in the protein precipitate is a function of the hydrogen ion concentration of the mixture but, in two experiments, it was found that the filtrate obtained by precipitating blood with a mixture containing 5 per cent nitric acid and 0.6 per cent picric acid gave values that agreed well with those obtained by precipitation with picric acid alone or by oxidation of the whole blood with nitric acid and that were considerably higher than those obtained by the use of picric acid alone. Evidently, some protein precipitants do completely displace the chlorine from combination with the protein, in spite of a high hydrogen ion concentration, whereas others do not.

BIBLIOGRAPHY.

1. Greenwald, I., *J. Biol. Chem.*, 1919, xxxviii, 439.
2. Greenwald, I., and Gross, J., *Proc. Soc. Exp. Biol. and Med.*, 1919–20, xvii, 50.
3. Bruhns, G., *Z. physiol. Chem.*, 1890, xiv, 533.
4. von Korányi, A., *Z. klin. Med.*, 1897, xxxiii, 1.
5. Myers, V. C., and Short, J. J., *J. Biol. Chem.*, 1920, xliv, 47.
6. Austin, J. H., and Van Slyke, D. D., *J. Biol. Chem.*, 1920, xli, 345.
7. Gazzetti, C., *Arch. fisiol.*, 1912–13, xi, 81.
8. Rappleye, W. C., *J. Biol. Chem.*, 1918, xxxv, 509.
9. Oppler, B., *Z. physiol. Chem.*, 1910–11, lxx, 198.
10. Foster, G. L., *J. Biol. Chem.*, 1917, xxxi, 483.
11. Smith, M., *J. Biol. Chem.*, 1920–21, xiv, 437.
12. Dugardin, M., *Ann. chim. anal.*, 1918, xxiii, 59.
13. Rodillon, G., *Presse méd.*, 1920, xxviii, 85.
14. Wetmore, A. S., *J. Biol. Chem.*, 1920–21, xlv, 113.
15. Harding, V. J., and Mason, E. H., *J. Biol. Chem.*, 1917, xxxi, 55.
16. Richter-Quittner, M., *Biochem. Z.*, 1919, xcv, 179.
17. Rieger, J. B., *J. Lab. and Clin. Med.*, 1920–21, vi, 44.
18. Whitehorn, J. C., *J. Biol. Chem.*, 1920–21, xlv, 449.

THE COMBINED URIC ACID IN BEEF BLOOD.

By ALICE ROHDE DAVIS, ELEANOR B. NEWTON, and STANLEY R. BENEDICT.*

Some years ago one of us[1] reported the occurrence of large amounts of uric acid in the corpuscles of beef blood, and evidence was offered to show that this uric acid existed in a stable combination from which it could be liberated by boiling with hydrochloric acid, or through enzyme action of the blood. In the present paper we are able to report the isolation and identification of the compound of uric acid which exists in beef blood.

For isolation of the compound of uric acid the following procedure has been employed. Fresh defibrinated beef blood is poured slowly into 5 volumes of boiling 0.01 N acetic acid and the mixture allowed to boil for about a minute and then poured on to a filter. The straw-colored filtrate obtained is boiled down to the volume of the original blood, chilled, and treated with one-tenth volume of colloidal iron (Merck's 5 per cent solution of dialyzed iron) to remove the last traces of protein. The precipitate obtained here is filtered off and the clear filtrate treated with an equal volume of 0.5 per cent solution of mercuric acetate. This mixture is allowed to stand for from 12 to 24 hours at room temperature. The light yellow flocculent precipitate which slowly forms contains all the free uric acid present in the original blood, together with a small amount of the desired uric acid compound. After filtering off the precipitate an equal volume of 20 per cent crystallized sodium acetate solution is added to the

[1] Benedict, S. R., *J. Biol. Chem.*, 1915, xx, 638.

perfectly clear filtrate. A precipitate forms slowly which is identical in appearance with the one already removed. After allowing the mixture to stand for 48 hours the supernatant fluid is decanted and the precipitate collected and washed in centrifuge tubes. The washing with distilled water is accomplished by thoroughly stirring up the precipitate each time before centrifugation, and afterward decanting the fluid. This process is continued until the precipitate fails to separate completely after centrifuging for 20 minutes. The precipitate (which contains a mercury compound of the uric acid compound and apparently very little other material) is then suspended in hot water and decomposed with hydrogen sulfide. The precipitate is filtered off and the residue washed with a little hot water. The filtrate is then concentrated under diminished pressure at from 40-45° until crystals separate. The material is allowed to stand over night and the crystals are filtered off next morning, washed with alcohol and ether and dried. The yield from a sample of 15 liters of blood is about 0.6 gm. of practically pure crystals. The product is recrystallized from a small quantity of boiling water. Thus obtained the compound crystallizes in colorless square plates (Fig. 1). When the hot solution is rapidly cooled the compound sometimes separates in flat needles, which on standing in contact with the mother liquor are completely transformed into the typical square plates. Even very small amounts of impurity may prevent crystallization for a longer or shorter period. In such instances, if not too dilute, the solution is very apt to form a water-clear gel. Such a gel may crystallize spontaneously, or the process may be hastened by seeding with a little of the crystalline material. The compound is very difficultly soluble in cold water, but dissolves in boiling water, and is insoluble in alcohol and ether. It is acid in reaction and dissolves readily in alkali. The compound is not precipitated by silver magnesia mixture and gives no reduction test with alkaline copper solutions. When tested for uric acid by the older phosphotungstic acid method the compound yields a quantity of color equivalent to that given by one-fifth of its weight of pure uric acid. When tested by the new cyanide method proposed by one of us[2] the substance yields an amount of color equivalent to that given by one-ninth of its weight of pure uric acid. On distillation with

[2] Benedict, S. R., *J. Biol. Chem.*, 1922, li, 187.

12 per cent hydrochloric acid the aniline test for furfural is positive. After hydrolysis the compound also yields a strongly positive test for pentoses with orcin.

Fig. 1. Photomicrograph of the crystalline compound of uric acid and pentose from beef blood.

The compound is apparently a monobasic acid. The optical activity of the sodium salt (prepared only in solution) was found to be

$$[\alpha]_D = +20.42°$$

No water of crystallization was found in the compound when heated *in vacuo* for 18 hours surrounded by the vapor from

boiling toluene. When heated to somewhat above 300° the compound does not melt. Heating with strong sulfuric acid leads to charring and the odor of caramel.

Early investigation of the compound showed that it is made up of uric acid and a carbohydrate group in the proportion 1:1. Identification of the carbohydrate portion has been difficult on account of the small quantities of material available, and because the original compound is so stable toward acid that the sugar is apt to be destroyed during the hydrolysis. After studying the conditions of the hydrolysis very closely we have been able to obtain data concerning the sugar present which we believe serve to establish its identity beyond reasonable doubt.

0.24 gm. of the compound was boiled for 7 hours in 40 cc. of 0.10 N sulfuric acid under a reflux condenser. As the process of hydrolysis proceeded uric acid separated. The mixture was allowed to stand over night and the uric acid was filtered off. The filtrate was then concentrated under diminished pressure to small volume. The uric acid was again removed (by centrifugation this time) and the clear and almost colorless solution exactly neutralized with sodium carbonate. The solution was then read in a polariscope, and a portion of it used for a quantitative sugar determination by means of the picric acid-picrate method. The angle of rotation obtained for the sugar was

$$[\alpha]_D^{2} = -20.06°$$

The angle of rotation of d-ribose[3] was then determined using the same method to estimate the sugar quantitatively. The angle obtained was

$$[\alpha]_D^{2} = -19.62°$$

Another portion of the hydrolyzed solution was used for the preparation of a phenylosazone. This was obtained in amorphous form and purified by dissolving in acetone and precipitating with water. The osazone of the unknown sugar, an osazone prepared from d-ribose, and a mixture of these two, when heated together in the same bath melted together at 149° (uncorrected). Very

[3] We are indebted to Dr. P. A. Levene for furnishing us with a sample of pure d-ribose.

rapid heating of the bath raised the melting points of both the osazones practically identically.

After hydrolyzing a known amount of compound the sugar obtained (determined by the picrate method, using ribose as standard) amounted to 101 per cent of the theoretical. Uric acid set free under similar conditions was identified by elementary analysis (see below) and was determined quantitatively by the color reaction. This determination gave a uric acid content for the original compound of 51.5 per cent found against 52.8 per cent calculated for a compound containing equal molecules of uric acid and d-ribose minus 1 molecule of water.

Elementary analyses of the compound are not very satisfactory. This is probably due, at least in part, to the fact that we used quite small quantities in making these determinations. The figures obtained were

Uric acid + pentose − 1 H_2O.
Calculated.   C 39.9, H 3.9, N 18.6.
Found.   " 39.1, " 5.4, " 18.0.

Analysis of the uric acid yielded by hydrolysis of the compound gave the following figures.

Calculated.   C 35.6, H 2.3, N 33.3.
Found.   " 35.5, " 3.3, " 33.1.

We feel warranted in concluding that the uric acid compound in beef blood is made up of 1 molecule of uric acid plus 1 molecule of d-ribose minus 1 molecule of water.

The biological properties of the uric acid compound are being investigated.

THE DISTRIBUTION OF THE COMBINED URIC ACID IN THE CORPUSCLES OF BEEF BLOOD.

By ELEANOR B. NEWTON and ALICE ROHDE DAVIS.

(From the Laboratory of the Boston Psychopathic Hospital, and the Department of Biological Chemistry, Harvard Medical School, Boston.)

Benedict[1] has reported the presence of combined uric acid in beef blood and has shown by quantitative colorimetric determinations that the total uric acid, free and combined, is contained in the corpuscles. The present work was planned to determine whether the uric acid is present chiefly in the erythrocytes or in the leucocytes.

Fresh defibrinated beef blood was centrifuged at high speed for $\frac{1}{2}$ hour, the clear serum pipetted off, and the film of white cells lifted from the red cells. The proteins of the serum and the red cells were removed by pouring each material into boiling 0.01 N acetic acid, the filtrates from the coagulum concentrated to the original volumes, cooled, and treated with colloidal iron in the usual way for the removal of protein from whole blood in the Benedict[2] modification of the Folin-Denis procedure for the determination of uric acid colorimetrically in blood. The white cells were quickly rinsed once and suspended in a small amount of distilled water and the film was ground with sand. This mixture of white cells and sand was then washed into boiling acetic acid, the final acidity of which was approximately 0.01 N. The filtrate from sand and protein was concentrated, boiled with aluminium cream, and again filtered. Clear filtrates were in all cases obtained. The free uric acid was determined by the procedure cited above,[2] and the combined uric acid after hydrolysis as carried out by Benedict.[3] To assure us that neither free nor

[1] Benedict, S. R., *J. Biol. Chem.*, 1915, xx, 638.

[2] Benedict, S. R., *J. Biol. Chem.*, 1915, xx, 629.

[3] Benedict, S. R., *J. Biol. Chem.*, 1915, xx, 634.

combined uric acid had been removed with the proteins of the
white cells a sample of white cells was hydrolyzed directly omitting
the protein removal and then submitted to a determination for
uric acid in the usual way.

TABLE 1.

Sample No.	Quantity of blood.	Material.	Uric acid in part analyzed Calculated per 100 cc. of blood.	Remarks.
	cc.		mg.	
I	20	Whole blood.	5.1	Hydrolyzed filtrate.
I	20	" "	4.8	" "
I	40	Red cells.	4.7	" "
I	40	" "	4.5	" "
I	50	White cells.	0	" "
I	50	" "	0	" "
I	100	" "	0	" without removing protein.
I	40	Serum.	0	Hydrolyzed filtrate.
I	40	"	0	" "

CONCLUSION.

Since no uric acid could be detected in the amounts of white
cells or of serum used and the figure for total uric acid from the
red cells compared closely with that for whole blood we conclude
that the combined uric acid is contained in the erythrocytes.

COMBINED URIC ACID IN HUMAN, HORSE, SHEEP, PIG, DOG, AND CHICKEN BLOOD.

By ELEANOR B. NEWTON and ALICE ROHDE DAVIS.

(From the Laboratory of the Boston Psychopathic Hospital, and the Department of Biological Chemistry, Harvard Medical School, Boston.)

INTRODUCTION.

The isolation and identification of combined uric acid in beef blood by Davis, Newton, and Benedict[1] as *d*-ribose and uric acid less 1 molecule of water has led to the study of the distribution of combined uric acid in several species of animals. By quantitative colorimetric determinations of uric acid before and after hydrolysis of a protein-free blood filtrate as well as before and after enzyme action Benedict[2] was led to believe that combined uric acid existed in other species.

The isolation of the compound has been undertaken by the original procedure for beef blood in the hope that even where the quantities of combined uric acid were so minute as to escape detection by the method of hydrolysis and quantitative determination of uric acid they would appear in the isolation. At the final stage of the isolation procedure a non-crystalline residue was hydrolyzed and tested colorimetrically for the pentose and for the uric acid radicals of combined uric acid.

EXPERIMENTAL.

Fresh defibrinated blood was deproteinized with heat and acetic acid, followed by treatment in the cold with colloidal iron. The protein-free filtrates were concentrated to the original blood volume and the material was treated with an equal volume of 0.5 per cent mercuric acetate to remove uric acid. The filtrates

[1] Davis, A. R., Newton, E. B., and Benedict, S. R., *J. Biol. Chem.*, 1922, liv, 595.

[2] Benedict, S. R., *The Harvey Lectures*, 1915-16, xi, 346.

were then treated with an equal volume of 20 per cent sodium acetate solution. An aqueous suspension of these mercury precipitates was then freed from mercury by decomposing with hydrogen sulfide, and filtering. The filtrates were concentrated under reduced pressure to a few drops or to 2 to 3 cc. and a slightly colored, more or less thick, fluid was obtained. If crystals did not appear, this residue, suspected of containing dissolved combined uric acid was hydrolyzed and distilled from 12 per cent hydrochloric acid to split off uric acid and to form furfural from pentose. The distillate was tested with aniline acetate paper for furfural and the residue, after neutralization with sodium carbonate, was tested for uric acid. In some cases a caramel odor was noted in the residue after distillation. Only rough estimations of the amounts of combined uric acid could be made by this method.

Two experiments were carried out on human blood. 360 cc. of a mixture of blood 1 to 5 days old, obtained from pathological cases, were employed in one experiment. Clear filtrates and typical mercury precipitates were obtained. No crystals appeared but the residue gave positive tests for pentose and for uric acid.

600 cc. of fresh defibrinated human blood from three normal individuals were later obtained from which a small amount of white, crystalline material was isolated, and several times recrystallized. The crystals were typical in form, but the quantity was too small to weigh. They were therefore distilled with 12 per cent hydrochloric acid. A very brilliant pentose test was here secured.

1,000 cc. of fresh defibrinated horse blood yielded a brownish, partly crystalline residue, a strong caramel odor on hydrolysis, and positive tests for pentose and for uric acid.

3,500 cc. of fresh defibrinated sheep blood yielded a gelatinous residue with a few crystals, which residue on hydrolysis gave positive pentose and uric acid reactions.

3,000 cc. of fresh defibrinated pig blood yielded a straw-colored fluid with no crystals, but gave positive pentose and uric acid reactions.

1,200 cc. of fresh defibrinated dog blood yielded a straw-colored fluid with no crystals, but gave positive pentose and uric acid reactions.

460 cc. of fresh defibrinated chicken blood gave a very light colored fluid with no crystals, but positive pentose and uric acid reactions.

CONCLUSION.

Evidences of the presence of combined uric acid have been found in each species of animal studied. The quantity in beef blood far exceeds that in any other animal blood so far analyzed. Next in quantity would appear to be that of human blood. Only traces were discernible by this procedure in the blood of the horse, sheep, pig, dog, or chicken.

THE INFLUENCE OF WATER DEPRIVATION, PILOCARPIN, AND HISTAMINE UPON CHANGES IN BLOOD CONCENTRATION IN THE RABBIT.

By FRANK P. UNDERHILL and S. CLEMENT ROTH.

(From the Department of Pharmacology and Toxicology, Yale Univeristy, New Haven.)

In various fields of clinical medicine symptoms and serious disorders, which are often attributed to unknown pathogenic factors, such as toxins, may be due entirely to a negative water balance of the body. That changes in blood concentration indicate this dehydration has been pointed out by Underhill and his collaborators (1 to 6). The possible ways in which such an effect may be brought about are very numerous, and many of the possibilities of concentrated blood in various diseased conditions undoubtedly remain unrecognized. Every addition to our knowledge relative to the mechanism of water balance in the organism brings the ultimate solution of the problem closer at hand and opens the way for better and more rational treatment of some of the obscure phases of disease.

In the present communication water changes in the blood have been studied in the rabbit under the influence of water deprivation, and the introduction of pilocarpin and histamine. The rabbit has been chosen in this instance for the reason that it is more or less refractory to various influences to which the dog is susceptible, and thus extends the range of factors involved in regulation of water balance.

Rabbits were maintained in a fasting condition unless otherwise specified.

Methods.—The methods employed were those usually followed in this laboratory for work of this character; hemoglobin estimation by the procedure of Cohen and Smith being employed as a criterion of changes in blood concentration. Injections were made into an ear vein.

Water Deprivation.

Previous (7) experience with water deprivation in the fasting rabbit has shown that water withheld for a period of 4 or 5 days

CHART 1. Rabbit 57. The influence of water deprivation upon blood concentration.

CHART 2. Rabbit 84. The influence of water deprivation upon blood concentration.

produces little or no change in blood concentration. The present experiments of which illustrations are given in Charts 1, 2, and 3, representative of many protocols, confirm this observation and

further show that after 5 to 7 days of water deprivation blood concentration becomes significantly increased. Charted with the alteration in blood concentration is the percentage loss of body weight.

CHART 3. Rabbit 101. The influence of water deprivation upon blood concentration.

CHART 4. Rabbit 102. The influence of water deprivation upon blood concentration.

It is a noteworthy fact that blood concentration in these experiments was not held uniformly at a level considered dangerous by Underhill (2); namely, 125 per cent of the normal value. A similar result has been obtained in other connections, Underhill and Errico (5), Underhill and Ringer (3), and Underhill and Kap-

sinow (6). The rapidity with which blood concentration resumes
the normal level in the rabbit after fluid intake is well illustrated
in Charts 1, 3, and 4. This result is confirmatory of observations
in the dog by Keith (8) and Underhill and Kapsinow (6).

In many of the experiments of this nature the animals died even
though water was supplied on the day preceding death. Death
under these circumstances might be referred to the irreparable
damage done to the tissues by the body's desiccation. Such a
conclusion should not, however, be drawn too hastily since fasting
rabbits with water freely supplied die in about the same length of
time. It is therefore obvious that to ascribe water deprivation
as the sole cause of death under the experimental conditions would
be erroneous.

Pilocarpin.

The loss of water in the rabbit by the use of pilocarpin is visible
in the profuse secretion of saliva and in the watery character of the
stools. By bringing about a rapid water elimination in this animal
pilocarpin served as a useful means of causing marked changes in
blood concentration which are represented graphically in Charts
5 to 9. The relative ease with which it is possible to effect a
change in blood concentration of approximately 15 per cent in
rabbits may be seen from Charts 5 to 8. In Chart 5 the animal
had been well fed up to the time of experiment whereas in Charts
6 and 8 water and food had been withheld for periods of 1 and 2
days, respectively. Pilocarpin causes rapid rises in blood concen-

CHART 5. Rabbit 1. Food and water. The influence of pilocarpin upon
blood concentration.

CHART 6. Rabbit 2. Without food and water for 1 day. The influence of pilocarpin upon blood concentration.

CHART 7. Rabbit 3. Without food and water for 1 day. The influence of pilocarpin upon blood concentration.

CHART 8. Rabbit 4. Without food and water for 2 days. The influence of pilocarpin upon blood concentration.

CHART 9. Rabbit 9. Without food and water for 10 days. The influence of pilocarpin upon blood concentration.

tration which are followed by equally rapid reductions in concentrations. Evidently the water of the tissues, as represented by alterations of water content of the blood, is sufficient to prevent anhydremia and the subsequent vicious circle. The water adjustment, however, does not take place so quickly that it cannot be detected. The relative rate at which the blood returns to normal will depend upon the degree of desiccation of the body previous to the experimentation, and the length of time the abnormal water loss induced by pilocarpin continues.

The extent to which desiccation of the tissues of a rabbit can take place is shown in Chart 9, the animal being without food and water for a period of 9 days. This rabbit survived the drastic disturbances in its water equilibrium in spite of the high level of blood concentration attained under pilocarpin treatment. This experiment also illustrates the rapidity with which blood concentration is reduced under the influence of water introduction.

Histamine.

In dogs histamine produces a marked change in blood concentration. Since rabbits are in general somewhat refractory to histamine effects the influence of the drug upon blood concentration has been tested in this animal. The observations made show that histamine invariably causes a slight decrease in concentration of the blood rather than an increase (see Charts 10, 11, and 12), the animal thus conforming to its general peculiarities with respect to histamine action.

The general effects upon the rabbit of histamine intravenously introduced fall into one of two groups. In the first place death may occur with violent inspiratory efforts within a minute, or secondly a train of symptoms ensue followed always by complete recovery. The respiration becomes rapid, the ears are cold, and a moderately deep narcosis sets in. Defecation and urination may occur, and the attitude of the animal is sprawling with the head sunk on the table. During this period it is almost impossible to obtain blood from the ears in sufficient quantity for hemoglobin estimation. A similar subcutaneous injection has only a slight influence, if any. With recovery it becomes easier to obtain blood from the ear. Within half an hour subsequent to histamine

administration the animal suddenly raises itself from its sprawling position and resumes a normal condition.

CHART 10. Rabbit 201. The influence of histamine upon blood concentration.

CHART 11. Rabbit 202. The influence of histamine upon blood concentration.

CHART 12. Rabbit 203. The influence of histamine upon blood concentration.

Attention is directed to Chart 13 illustrating an experiment in which a rabbit without food for 8 days and water for 9 days

received an injection of histamine. No influence upon blood concentration could be seen during the period of observation.

CHART 13. Rabbit 206. Without food and water for 10 days. The influence of histamine upon blood concentration.

SUMMARY.

Complete deprivation of water leads to blood concentration in rabbits. The level attained by the concentration lies within the limits of safety suggested by previous investigations.

High blood concentration induced by water deprivation rapidly falls to normal levels upon administration of water.

Under the influence of pilocarpin blood concentration is markedly increased but quickly returns to the normal level. Pilocarpin action superimposed upon prolonged water deprivation causes exceptionally high blood concentrations which rapidly attain the normal upon water administration.

In the rabbit histamine fails to produce blood concentration. Little or no change in blood concentration is to be observed except perhaps an insignificant lowering. These observations with histamine are quite unlike those previously obtained under similar experimental conditions with the dog.

BIBLIOGRAPHY.

1. Underhill, F. P., The lethal war gases: physiology and experimental treatment, New Haven, 1920.
2. Underhill, F. P., *The Harvey Lectures*, 1918-19, xiv, 234.
3. Underhill, F. P., and Ringer, M., *J. Am. Med. Assn.*, 1920, lxxv, 1531.
4. Underhill, F. P., and Ringer, M., *J. Pharmacol. and Exp. Therap.*, 1922, xix, 163.
5. Underhill, F. P., and Errico, L., *J. Pharmacol. and Exp. Therap.*, 1922, xix, 135.
6. Underhill, F. P., and Kapsinow, R., *J. Biol. Chem.*, 1922, liv, 459.
7. Bogert, L. J., Underhill, F. P., and Mendel, L. B., *Am. J. Physiol.*, 1916, xli, 189.
8. Keith, N. M., *Am. J. Physiol.* 1922, lix, 452.

THE USE OF IODINE IN THE DETERMINATION OF GLUCOSE, FRUCTOSE, SUCROSE, AND MALTOSE.

By F. A. CAJORI.

(From the Department of Chemistry, Stanford University, California.)

INTRODUCTION.

Almost without exception, the available methods for the quantitative determination of individual sugars, in solutions where several of them are present together, involve the use of the polariscope. With sugar solutions of low concentration, the observed rotation may be only a few angular degrees, and results based on such slight differences are far from accurate. Again, highly colored solutions, such as are often obtained from plant material, must be clarified before they can be used in the polariscope and not inconsiderable loss of sugar by adsorption occurs during the clarification process. Moreover, solutions of plant origin often contain other optically active substances.

Prerequisite to an understanding of the manner of carbohydrate formation in the photosynthetic process is an accurate picture of the quantitative relations that exist between the various sugars of the leaf. Advance in this field is largely dependent upon the development of analytical methods, as the analysis of plant material presents many extraordinary difficulties not encountered in material of animal origin.

Romijn (1) and Bougault (2) observed that glucose in alkaline solution is completely oxidized to gluconic acid by an excess of iodine and that under the same conditions fructose and sucrose are unchanged. This would seem to offer the basis for a method of distinguishing between the individual sugars without recourse to the use of the polariscope. These two authors recognized the possibilities of the use of iodine as a reagent for differentiating between aldose and ketose sugars, and they suggested methods

617

involving its use. Several other investigators have reported their experiences with iodine as an oxidizing agent for the quantitative determination of sugars with varying success.

In the following work we have endeavored to define more sharply the conditions under which sugars are oxidized by iodine, and, on the basis of these observations, have developed a method for the determination of glucose, fructose, sucrose, and maltose when they occur together.

Romijn (1) was unable to obtain satisfactory quantitative results from the oxidation of glucose by iodine with sodium hydroxide or potassium hydroxide and suggested the use of sodium borate. With this weak alkali, 18 to 40 hours were required to complete the oxidation of glucose to gluconic acid. Willstätter and Schudel (3) pointed out the need of sufficient amounts of alkali to neutralize the acid formed in the reaction. With an excess of 0.1 N sodium hydroxide, and oxidation with iodine for 15 minutes, they were able to recover quantitatively the glucose present in invert sugar.

Bougault (2), using sodium carbonate, observed a quantitative oxidation of glucose by iodine in 30 minutes. Maltose, lactose, arabinose, and other aldose sugars were found by him to be oxidized by iodine. Colin and Liévin (4) modified Bougault's procedure, using disodium phosphate instead of sodium carbonate. They found that 1 hour was the time required for the oxidation of the glucose, with this modification.

Lately, Judd (5) has published the results of a critical study of these methods. She was unable to confirm Romijn's or Bougault's findings that iodine oxidizes glucose to gluconic acid, and that under the same conditions fructose is not oxidized at all. She ascribed this failure, in part, to possible enolization of the sugars in the presence of alkalies. Judd believed, however, that valuable methods for the analysis of glucose-fructose solutions could be developed by utilizing the reducing power of the sugar solution on iodine, as well as its reducing power on copper solutions. In contrast to the findings of Judd, Baker and Hulton (6), using Willstätter's procedure, experienced no difficulty in recovering glucose as gluconic acid after 5 minutes oxidation with iodine.

EXPERIMENTAL PART.

Preliminary Experiments with Glucose and Fructose.—The reaction between glucose and an excess of iodine in a solution made alkaline with sodium carbonate was followed by determining the amount of iodine present in aliquot parts of the oxidation mixture at intervals during the course of the oxidation. The amount of iodine present was determined by titration with standard sodium

thiosulfate after having made the solution slightly acid, using
soluble starch as an indicator. An approximately 0.1 N iodine
solution, standardized with 0.05 N sodium thiosulfate was used.
The 0.05 N sodium thiosulfate was prepared from a normal stock
solution and standardized in the usual way with the aid of 0.1 N
potassium permanganate. The preliminary trials were carried
out in a thermostat at 25°C. Later it was found that the velocity
of the reaction was not noticeably changed at room temper-
atures (18−22°). Blank determinations were made, using distilled
water instead of a sugar solution, but in all other respects the
same conditions were maintained. Typical results of these trials,
expressed in quantities of iodine present in the aliquot parts,
are given in Table I.

TABLE I.

Reduction of Iodine by Glucose and Fructose.

Time of oxidation.	Iodine present in 20 cc. aliquot parts.			
	Glucose solution.	Blank.	Fructose solution.	Blank.
min.	*mg.*	*mg.*	*mg.*	*mg.*
0	123.0	123.3	168.8	182.9
10	94.0	122.9	168.1	181.4
20	92.4	122.3	167.7	181.4
30	93.3	123.3	167.7	182.0
45	93 3	122.9	167.3	178.6
60	92.6	122.5	167.3	
90	92.7	122.5	166.8	181.4

These results show that iodine is rapidly reduced in the pres-
ence of glucose, the reduction, apparently, being completed at
the end of 20 minutes. On the other hand, there is no evidence
of any reduction of iodine by fructose. These results confirm
the findings of Romijn and Bougault. As indicated from the
analysis of the blanks, there are always small losses of iodine
from the solution during the course of the oxidation. That such
losses do occur is not surprising, considering the volatile nature of
iodine. Bougault noticed that on long standing more iodine disap-
peared from his solutions than could be accounted for by the theo-
retical amount required to oxidize the sugar to the mono-basic
acid. He ascribed this to a secondary oxidation of the hydroxyl
groups of the sugar. He reports no blank determinations, and it

seems much more reasonable to recognize the possibility of a mechanical loss of iodine than to assume oxidation of the hydroxyl groups of the sugars.

The apparent increase in the concentration of the iodine noted in the solutions during the second half hour of the oxidation is undoubtedly due to the liberation of iodine from the potassium iodide used in the iodine solution.

Bougault emphasized the necessity of having an excess of iodine present in order to insure complete oxidation of the glucose. We also found this an important condition to be maintained. The amount of iodine to be used depends in part on the alkalinity of the solution. In Table II are given some of our experiments

TABLE II.

Oxidation of Glucose as Influenced by Varying Amounts of Iodine and Sodium Carbonate.

Experiment No.	Time of oxidation.	Glucose present.	Iodine required to oxidize sugar.	Iodine present.	Glucose oxidized.	Na_2CO_3 present.	Ratio of iodine present to iodine required.
	min.	*mg.*	*mg.*	*mg.*	*mg.*	*per cent*	
1	25	16.2	22.8	186	15.8	3.7	8 : 1
2	25	10.8	15.2	124	10.6	1.5	8 : 1
3	25	21.7	30.6	186	21.1	1.4	6 : 1
4	25	22.0	31.0	125	21.8	0.8	4 : 1
5	25	48.7	68.5	186	48.4	2.1	2.7 : 1
6	25	48.4	68.0	186	48.1	1.0	2.7 : 1
7	25	48.4	68.0	124	45.4	1.1	1.8 : 1

where varying amounts of iodine and sodium carbonate were used.

It will be seen that with a sodium carbonate concentration of 1 or 2 per cent, three times the amount of iodine necessary to oxidize the sugar gives a complete reaction in 25 minutes. Twice the amount (1.8), as illustrated in Experiment 7, is not a large enough excess and incomplete oxidation resulted. In Experiment 1, the high concentration of sodium carbonate diminished the apparently large excess of iodine and again the glucose was incompletely oxidized.

We failed in attempts to oxidize glucose with iodine in neutral solution, but beyond having alkali present, and present in suffi-

cient quantities to neutralize the acid formed in the reaction, no
advantage could be observed in increasing the concentration of
the alkali. It is highly desirable to carry out the oxidations in
solutions of low alkalinity because of the effect of alkalies on
sugars. We finally chose for the oxidations, solutions with 1.0
to 1.5 per cent of sodium carbonate. Nef (7) reported that no
enolization of glucose took place with this strength of sodium
carbonate. The use of sodium carbonate instead of sodium
hydroxide seemed desirable in order to avoid any possible enoli-
zation of the sugars present.

Witzemann (8) found that disodium phosphate catalyzes the
oxidation of glucose by hydrogen peroxide. In trials where we
used 0.5 M Na_2HPO_4, we were unable to note any increase in the
velocity of the reaction between glucose and iodine that could

TABLE III.

Analysis of a Glucose Solution.

Glucose solution used	Glucose present.	Iodine reduced	Glucose found.	Molar ratio of iodine used to glucose present.
cc.	mg.	mg.	mg.	
3	10.37	14.1	10.00	1.92 : 1
6	20.74	28.6	20.29	1.96 : 1
8	27.65	38.8	27.52	1.99 : 1
10	34.56	48.5	34.41	1.99 : 1
14	48.38	67.5	47.89	1.98 : 1

be ascribed to any catalytic influence of disodium phosphate.
We also endeavored to catalyze the oxidation of glucose by
iodine by adding traces of ferrous chloride to the oxidation mix-
ture, but obtained no evidence of any influence of this iron salt
on the velocity of the reaction. Within limits, dilution of the
iodine solution has no effect on the rate or completeness of the
oxidation of glucose. Provided there is sufficient iodine present,
its concentration does not seem to be significant.

Analysis of Glucose Solutions.—The preliminary experiments
indicated that under suitable conditions iodine could be used
as a reagent for the quantitative estimation of glucose. That
this conclusion was justified is shown in Table III, where the
results of the analysis of a number of glucose solutions are given.
The protocol of a single experiment is given to illustrate the pro-

cedure that we adopted for the oxidation of glucose by iodine. The glucose used was a Bureau of Standards preparation and before using was dried *in vacuo* over phosphorus pentoxide.

To 10 cc. of a glucose solution, containing 34.56 mg. of glucose, 2 cc. of a 15 per cent Na_2CO_3 solution were added; 15 cc. of a 0.1 N iodine solution were then added, and the flask was stoppered and placed in the dark at room temperature for 25 minutes. Slightly more 10 per cent H_2SO_4 than that needed to neutralize the Na_2CO_3 was added and the iodine titrated immediately with 0.05 N $Na_2S_2O_3$. From the titration, 3.82 cc. of 0.1 N iodine, or 48.5 mg., had been reduced. Calculated for glucose, 34.41 mg.

Within the experimental error, the molecular ratio of the iodine reduced to the glucose present is 2 to 1. In other words, the reaction proceeds to gluconic acid according to the equation:

$$C_6H_{12}O_6 + 2I + H_2O \rightarrow C_6H_{12}O_7 + 2HI$$

The amount of iodine reduced by the sugar may then be used as a measure of the amount of glucose present.

Determination of Glucose in the Presence of Fructose and Sucrose. —The oxidation of glucose by iodine proceeds in the presence of small or large amounts of fructose or sucrose, without being influenced by either sugar. In Table IV are given results of the determination of glucose when fructose or sucrose are also present.

Analysis of Glucose-Fructose-Sucrose Solutions.—Iodine oxidizes glucose but not fructose or sucrose. Cupric hydroxide oxidizes both glucose and fructose. The use of these two reagents, then, will enable a determination to be made of glucose and fructose when together in solution, or of glucose, fructose, and sucrose when these three sugars occur together. In the latter case, the determination of the reducing power of the sugar solution on both the iodine and copper reagents before hydrolysis of the sucrose, and the reducing power on either of the reagents after hydrolysis, will give data from which can be calculated the amounts of the individual sugars which are present.

We have successfully applied such a procedure for the determination of the three sugars. Oxidation with iodine was carried out as described in the analysis of the glucose solutions. The copper reagent that we have used was Benedict's reagent (9) and the technique followed was that lately described by Spoehr

(10) for the determination of small amounts of reducing sugars. Briefly stated, this technique consists in oxidizing the sugars by placing centrifuge tubes containing the sugar and copper reagent in a boiling water bath for 3 minutes, and removing the cuprous oxide by centrifuging; the residual cupric copper is determined by titrating the iodine, liberated from an excess of potassium iodide by the cupric copper, with sodium thiosulfate. Benedict's solution was calibrated in terms of glucose and fructose by determining the reducing power of solutions of glucose and fructose made up from very pure and dry samples of these sugars.

One of the difficulties encountered was the establishing of suitable conditions for the inversion of the sucrose. A few hours heating on the water bath with sufficient hydrochloric acid to

TABLE IV.

Determination of Glucose in the Presence of Fructose or Sucrose.

Glucose present.	Fructose present.	Sucrose present.	Glucose found.
mg.	*mg.*	*mg.*	*mg.*
21.6	12.7		21.3
10.8	12.7		10.5
10.8	25.3		10.7
48.7		10.5	48.4
5.4		5.3	4.9
10.8		105.1	10.6
17.4		26.3	17.2

make a 1 per cent solution will completely invert the sucrose present. However, when fructose was also present a slight coloring of the solution occurred during the heating, and the reduction of iodine or copper was greater than could be accounted for by the amounts of glucose present. Evidently, fructose in acid solution and at 100°C. is partly decomposed, yielding compounds which are oxidized by iodine and cupric hydroxide. We endeavored to hydrolyze the sucrose with 1 per cent hydrochloric acid at 25°C. Under these conditions, the inversion was incomplete even when allowed to stand over night.

It was finally found that 2 hours at 60°C. with 1 per cent hydrochloric acid effected complete hydrolysis of the sucrose. The solutions remained water-clear and there was no evidence of any decomposition of fructose at this temperature. These conditions,

then, were chosen as being suitable for the hydrolysis of sucrose in the presence of fructose.

TABLE V.

Determination of Sucrose in the Presence of Glucose and Fructose.

Sucrose present.	Glucose present.	Fructose present.	Glucose found.	Sucrose found	Conditions of hydrolysis.
mg.	mg.	mg.	mg.	mg.	
56.7	22.0	43.6	21.8		Before hydrolysis.
56.7	22.0	43.6		61.7	1 per cent HCl, 3 hrs. at 100°.
56.7	22.0	43.6		63.5	1 " " " 3 " " 100°.
56.7	22.0	43.6		59.8	1 " " " 3 " " 100°.
56.7	22.0	43.6		48.7	1 " " " 16 " " 25°.
40.8	12.1	29.3	12.6		Before hydrolysis.
40.8	12.1	29.3		39.2	1 per cent HCl, 2 hrs. at 60°.
40.8	12.1	29.3		38.8	1 " " " 4 " " 60°.
40.8	12.1	29.3		38.8	1 " " " 6 " " 60°.

TABLE VI.

Determination of Glucose, Fructose, and Sucrose.

Sugar solution used.	Iodine reduced.	Copper solution reduced.*	Glucose found.	Glucose and fructose as glucose.	Remarks.
cc.	mg.	cc.	mg.	mg.	
25	43.5		30.9		Before hydrolysis.
25	70.9		50.3		After "
5.09		4.10		9.75	Before "
2.59		3.98		9.47	After "

Calculated for Glucose, Fructose, and Sucrose.

	Present per cc.	Found per cc.
	mg.	mg.
Glucose.................................	1.26	1.23
Fructose.................................	0.57	0.59
Sucrose.................................	1.56	1.57†

* 1 cc. of Benedict's solution = 2.379 mg. of glucose and 2.149 mg. of fructose.

† Average of determinations by iodine and copper.

The results of the hydrolysis of sucrose under various conditions are given in Table V.

The results of an analysis of a solution containing glucose, fructose, and sucrose are presented in Table VI. Utilizing both

iodine and cupric hydroxide as oxidizing agents and carrying out the analysis according to the procedure and under the conditions that have been outlined above, the method would seem to be accurate for the determination of the individual sugars to within an error of 3 per cent.

Experiments with Maltose.—Maltose is oxidized by iodine, 2 molecules of iodine reacting with 1 molecule of the sugar. We have found that this oxidation proceeds somewhat more slowly than the oxidation of glucose by iodine. Where glucose was completely oxidized by iodine in 25 minutes, maltose required 35 minutes to be completely oxidized to the mono-basic acid.

Maltose is much more resistant to acid hydrolysis than is sucrose. We could observe no evidence of hydrolysis of maltose by 1 per cent hydrochloric acid at 60°C. at the end of 2 hours and even at the end of 24 hours at 60°C., the hydrolysis was incomplete. 3 hours heating at 100°C. with 1 per cent acid effects complete hydrolysis of maltose but such vigorous treatment has been found partly to decompose fructose if present in the solution. In order, then, to extend the iodine-copper method that has been outlined above to solutions that contain maltose in addition to the other sugars, other means than acid hydrolysis of the maltose would seem to be necessary. For this purpose we have used maltase prepared from fresh yeast by the method of Willstätter, Oppenheimer, and Steibelt (11). We found that maltose is completely hydrolyzed to glucose with yeast maltase after 3 hours digestion at 30°C. in a solution whose reaction has been adjusted to a pH of 6.1 to 6.8 with acid phosphate, according to the directions of Willstätter. The increase in the reducing power of the solution, as a result of the glucose formed in the reaction, may then be taken as a measure of the amount of maltose present.

Freshly prepared enzyme solutions must be used. After 2 or 3 days, the activity of the maltase was greatly diminished and proved to be unsatisfactory for a quantitative hydrolyis of maltose. Yeast maltase solutions contain substances that react with iodine, and in the calculations, based on the amount of iodine reduced as the result of the enzyme hydrolysis, it was found necessary to apply rather large corrections.

The results of the experiments with maltose are given in Table VII.

By the use of maltase solutions and adequate control experiments, planned to determine the activity of the maltase preparation, as well as the reducing power of this preparation on iodine and cupric hydroxide, it would seem possible to extend the iodine-copper method to include maltose in addition to the other sugars. With a mixture of sugars, the maltose, unchanged by acid hydrolysis of sucrose at 60°C., would be measured by the increase in glucose content of the solution after digestion with maltase.

TABLE VII.

Determinations of Maltose with Iodine under Various Conditions.

Experiment No.	Maltose present.	Iodine reduced.	Maltose found.	Glucose found.	Glucose calculated from $C_{12}H_{22}O_{11} \cdot H_2O$.	Conditions.
	mg.	*mg.*	*mg.*	*mg.*	*mg.*	
1	213.0	148.8	211.0			Oxidation with I for 35 min.
2	30.0	20.9	29.7			" " " " 35 "
3	16.7	11.7	16.7			" " " " 35 "
4	18.2	12.7	18.1			" " " " 35 "
5	18.6	13.2	18.7			" " " " 35 "
6	39.6	24.9	35.4			" " " " 35 "
7	39.6	24.9	35.4	0	0	1 per cent HCl, 2 hrs. at 60°.
8	30.0	47.8		34.0	30.0	Hydrolysis with maltase.
9	16.7	23.1		16.4	16.7	" " "
10	16.7	22.4		15.9	16.7	" " "
11	16.7	23.9		17.0	16.7	" " "

The results reported in this paper are based on the analysis of pure sugars. It is well to point out that in applying this method to sugar-containing fluids such as plant extracts that contain, in addition to the sugars, other compounds, the accuracy of the results will depend on the freedom of the solution analyzed from reducing substances other than sugars, or compounds that will react with iodine.

SUMMARY.

The oxidation of glucose by iodine has been investigated and the conditions whereby a quantitative oxidation of glucose to gluconic acid may be effected have been established.

Using iodine and cupric hydroxide as oxidizing agents, a method has been presented for the determination of glucose, fructose, and sucrose where these sugars occur together, and in small quantities.

Results suggesting that the method may be extended to include maltose are given.

The larger part of the experimental work reported in this paper was done in the Coastal Laboratory of the Carnegie Institution of Washington, at the suggestion and under the direction of Dr. H. A. Spoehr. The author is greatly indebted to Dr. Spoehr for his helpful advice and criticism, and to the Carnegie Institution for the privilege of working in the Coastal Laboratory.

BIBLIOGRAPHY.

1. Romijn, G., *Z. anal. Chem.*, 1897, xxxvi, 349.
2. Bougault, J., *J. pharm. et chim.*, 1917, series 7, xvi, 97; *Compt. rend. Acad.*, 1917, clxiv, 1008.
3. Willstätter, R., and Schudel, G., *Ber. chem. Ges.*, 1918, li, 780.
4. Colin, H., and Liévin, O., *Bull. Soc. chim.*, 1918, series 4, xxiii, 403.
5. Judd, H. M., *Biochem. J.*, 1920, xiv, 255.
6. Baker, J. L., and Hulton, H. F. E., *Biochem. J.*, 1920, xiv, 754.
7. Nef, J. U., *Ann. Chem.*, 1914, cdiii, 204.
8. Witzemann, E. J., *J. Biol. Chem.*, 1920–21, xlv, 1.
9. Benedict, S. R., *J. Biol. Chem.*, 1908–09, v, 485.
10. Spoehr, H. A., *Carnegie Inst. Washington, Pub. 287*, 1919, 31.
11. Willstätter, R., Oppenheimer, T., and Steibelt, W., *Z. physiol. Chem.*, 1920, cx, 232.

PIGMENTS OF THE MENDELIAN COLOR TYPES IN MAIZE: ISOQUERCITRIN FROM BROWN-HUSKED MAIZE.*

By CHARLES E. SANDO and H. H. BARTLETT.

(*From the Office of Physiological and Fermentation Investigations, Bureau of Plant Industry, United States Department of Agriculture, Washington, and the Department of Botany, University of Michigan, Ann Arbor.*)

INTRODUCTION.

At Cornell University, Emerson has been the leader in an important series of genetical investigations of maize, in which the Mendelian factorial composition of many color varieties has been established. In a recent memoir[1] he has dealt especially with what he terms plant colors; *i.e.*, colors due to pigments (other than those of the chloroplasts) which are commonly seen in the husks, the staminate inflorescence, the foliage generally, and the stem.

Of such plant colors he has established the genetical relations of six main types as follows: (1) purple, (2) sun red, (3) dilute purple, (4) dilute sun red, (5) brown, (6) green. In a former paper in this Journal[2] we have pointed out the importance of chemical studies of such color series as this in which the genetical factorial analysis has been made. Only by the chemical investigation of genetically known material may we hope to come to any satisfactory understanding of the meaning of the Mendelian analysis, and of the actual operation of the factors which in such an analysis are known as symbols.

The plant color series in maize immediately suggests a parallelism with similarly related varieties of other plants in which flower color (due to cell sap pigments of the flavonol and anthocyanin groups) varies from white to various shades of yellow, red, and purple. Such a parallelism has already been confirmed[3] by the isolation of a quercetin glucoside from

---

[1] Emerson, R. A., *Cornell Univ. Agric. Exp. Station, Memoir XXXIX*, 1921, 156.

[2] Sando, C. E., and Bartlett, H. H., *J. Biol. Chem.*, 1920, xli, 495.

[3] Sando, C. E., and Bartlett, H. H., *J. Agric. Research.*, 1921, xxii, 1.

brown-husked maize (Type 5) and by the occurrence of a corresponding anthocyanin (not yet isolated) in purple (Type 1).

In order that the geneticist may perceive the feasibility of a chemical attack on the problem of interpreting genetical factors, it does not seem out of place to state briefly the simple chemical relationship of the cell sap pigments to one another. Without some chemical knowledge of the pigments, the geneticist obviously cannot advance beyond the symbolic expression of his results.

The yellow sap pigments derived from flavone may be subdivided into two groups—flavone and flavonol. The flavone pigments are derivatives of the mother substance flavone (I), or phenyl-2-phenopyrone-4,[4] while the flavonol pigments are derivatives of flavonol (II), which is characterized by the fact that the hydrogen in the pyrone nucleus is replaced by hydroxyl. The anthocyanidin pigments are derivatives of phenyl-2-phenopyrylium (III).[5]

I. Flavone.   II. Flavonol.   III. Phenyl-2-pheno-
                                                            pyrylium.

Compounds containing this latter nucleus are characterized by the fact that they form difficultly soluble, well crystalline oxonium salts, consequently the natural anthocyanidins are described as oxonium chlorides, regardless of the fact that the coloring matters probably exist in the plant as combinations with organic acids.

In the plant, hydroxy and methoxy derivatives of the three classes of pigments exist as glucosides. The anthocyanidin glucosides are the naturally occurring anthocyanins. The anthocyanidins in the form of their oxonium salts may be looked upon as formed from the corresponding flavonols by three steps: (a) reduction (by the addition of 2 atoms of hydrogen), (b) combination with an acid, in the plant cell probably an organic acid, but in the laboratory generally hydrochloric acid, and (c) by the elimination of a molecule of water. The three steps representing the transition from flavonol to anthocyanidin may be illustrated by the formation of cyanidin chloride from quercetin. Quercetin is not only a typical flavonol and one which is found widely distributed in nature, but it is also the particular one which is concerned in the pigmentation of maize.

[4] The numbering of the rings is that indicated by Decker and von Fellenberg (Decker. H., and von Fellenberg, T., *Ann. Chem.*, 1907, ccclvi, 296).

[5] Decker, H., and von Fellenberg, T., *Ann. Chem.*, 1907, ccclvi, 281.

Quercetin (yellow).   Pseudobase (colorless).

Cyanidin chloride (red).

(For the sake of simplicity the above reaction shows the transformation from flavonol to anthocyanidin, both of which are aglykones or non-sugar products of the splitting of the glucoside molecule. The reactions in the plant are doubtless concerned with the glucosides, since the flavonol glucosides which occur in the plant are water-soluble and more easily reduced than the flavonols themselves.) The delicate equilibrium that must be maintained in a plant cell in order that one or another cell sap pigment may be present is indicated by the ease with which reversal of this last step takes place. Merely by treatment with hot water, pelargonidin chloride (the anthocyanin of the scarlet geranium is a glucoside of pelargonidin) passes into the colorless pseudobase, by the addition of 1 molecule of water and loss of hydrochloric acid. Alcoholic extracts of anthocyanins, although deeply colored at first, frequently become rapidly decolorized by this reaction, which may possibly be found to afford an explanation of the variation of some flowers from white to red,[6] which is known to depend, in *Primula sinensis* var. *rubra*, upon the temperature at which the flowers develop, the flowers being white at 30–35°C., and red at 15–20°C.

It is obvious that the chemical relationships of these plant pigments are such that relatively slight changes in the condition of the cell might lead to large visible effects.

In both flavonols and anthocyanidins there is a variable number of hydroxyl groups which are capable of being condensed with sugars. Compounds with glucose, rhamnose, and galactose are known. Others will doubtless be found. Depending upon the position of the substituted hydroxyls of any one flavonol or anthocyanidin, and also upon the particular carbohydrate which condenses with it to form the glucoside, there is an opportunity for the existence of a large number of isomeric pigments of somewhat different properties. With one, two, or more hydroxyls

[6] Klebs, G., *Jahrb. wissensch. Bot.*, Leipsic, 1906, xlii, 155. cf. Wheldale, M. The anthocyanin pigments of plants, Cambridge, 1916, 87.

substituted, there is also an opportunity for several series of isomers, although only a monoglucoside series and a diglucoside series are known. Finally, variation in the position of the hydroxyls among the several mother substances provides possibilities for still more stereoisomers. Reference to the work of Perkin and Everest[7] shows that many flavones and flavonols have already been isolated. Willstätter[8] and his students have made great advances in the chemistry of the anthocyanins.

The early interest was centered in the yellow plant pigments because of their value as dyes. Consequently the glucosides, of great importance from the genetical standpoint, have been less studied than the flavones and flavonols. In the recent work on the anthocyanins the glucosides have received relatively more attention, but the geneticist finds little in the past work on flavonol glucosides and anthocyanins that enables him to argue that any particular yellow pigment is the mother substance of a particular purple. It is merely inferred that the most easy transition, involving the least readjustment of the molecule, is the one that takes place in the plant. Thus, if the yellow color of a certain plant were known to be due to a glucoside of quercetin, and if a genetically related purple type of the same species existed, one might logically expect the purple color to be due to a homologous glucoside of cyanidin. Or, if both yellow and purple pigments existed in the same variety, under circumstances that made it appear likely that one pigment was the mother substance of the other, there would be ground for expecting an equally simple relationship. What little direct evidence there is that supports this view has been found by Everest,[9] who has shown the coexistence in purple-black pansies of the closely related pair myricetin ($C_{15}H_{10}O_8$) and delphinidin ($C_{15}H_{10}O_7$), the former yielding the latter by reduction. To render the proof more satisfactory one would wish to show not only the close correspondence between the flavonol and the anthocyanidin, but also a correspondence in the sugar and the position of its attachment. It is obvious that a large field is here thrown open for investigation.

No compounds are more widespread in plants than the yellow and purple pigments under consideration. The characters de-

[7] Perkin, A. G., and Everest, A. E., The natural organic colouring matters, London and New York, 1918.

[8] Willstätter, R., et al., Summarized in foot-note 7.

[9] Everest, A. E., Proc. Roy. Soc. London, Series B, 1913–14, lxxxvii, 444.

pending upon their presence, absence, or distribution have often been the subject of genetical research, for the reason that color characteristics are frequently due to allelomorphic factors. If not allelomorphic, the color factors are interacting and the Mendelian ratios are interpreted from this standpoint. Thus, it is frequently the case that a simple monohybrid ratio is obtained in crosses between yellow and purple varieties. The cross between purple and brown-husked maize affords a case in point. The first hybrid generation is purple. The second shows segregation of purple and brown in a 3:1 ratio.

Color characters may be mutually exclusive, not coexisting in the same plant or tissue, or non-exclusive, as in the case of flowers in which both yellow and purple pigment coexist. Distribution factors may, in the latter case, cause a definite color pattern, as in variegated purple and yellow pansies, or the pigments may be mixed. An example of the intimate association of yellow and purple (or blue) is possibly indicated by the green flowers of certain hybrids between purple and yellow alfalfa, in which the resultant color is green. (It is not certain, however, that plastid pigments are not here involved.)

It goes without saying that it would be a great gain to both the physiologist and the geneticist if the place of these pigments in metabolism were known, and if it were possible to trace the train of reactions by which the same mother substance gives rise to different pigments in organisms of different genetical constitution. Hypothetical chemical explanations of the allelomorphism of color characters have been proposed, but all require complete or partial verification. For example, it has been suggested by Wheldale[10] that, in the simplest case, there would be at least two factors responsible for anthocyanin formation in flowers, one, designated C, representing chromogen, and another, designated R, representing an enzyme which is supposed to act upon C with the production of color. It is generally believed that in some cases a flavonol glucoside may itself act as a chromogen for the formation of an anthocyanin, as in the case of the purple-black pansy already mentioned.

[10] Wheldale, M., The anthocyanin pigments of plants, Cambridge, 1916, 211.

The available evidence would seem to indicate that cases of extreme simplicity are not often to be expected. For example, a flavonol from blue flowered *Delphinium consolida* L. has been isolated and examined by Perkin and Wilkinson[11] and found to be kaempferol. Willstätter and Mieg[12] prepared an anthocyanidin from a purple variety of the same species, and named it delphinidin. The flavonol corresponding to delphinidin is not kaempferol, but myricetin. Here we have a lack of the correspondence such as Everest found in his pansy. It must be emphasized, however, that in the case of *Delphinium* the two investigators used different varieties, and that the genetical relations are totally unknown, and probably complex, for there is evidence from another species of *Delphinium* of the complexity of pigmentation in this genus, showing that even a yellow species without known blue or purple forms may be a genetically unresolved mixture of different yellow types. That Perkin and Pilgrim[13] found three flavonols in the yellow flowers of *Delphinium consolida*, chosen at random, is therefore not surprising.

Thus far no series of color types of known genetical constitution has been thoroughly examined chemically, although an excellent beginning in this work was made by Wheldale and Bassett.[14] Their work concerned *Antirrhinum*, from which they were successful in isolating flavones (not flavonols) from two yellow varieties. The deep yellow variety, with the Mendelian constitution YYii contained both luteolin and apigenin. A dominant factor, I, transformed the deep yellow into ivory, or very light yellow, the homozygous ivory type having the formula YYII, and containing only the pale colored apigenin. Other factors brought about the formation of two different anthocyanins, which were unfortunately not obtained in satisfactory condition for a determination of constitution or identity. Since Wheldale and Bassett did their work before the publication of Willstätter's brilliant researches, it is hoped that the *Antirrhinum* material may still be worked out satisfactorily. However, it must be noted that no

11 Perkin, A. G., and Wilkinson, E. J., *J. Chem. Soc.*, 1902, lxxxi, 585.

12 Willstätter, R., and Mieg, W., *Ann. Chem.*, 1915, cdvii, 61.

13 Perkin, A. G., and Pilgrim, J. A., *J. Chem. Soc.*, 1898, lxxiii, 267.

14 Wheldale, M., and Bassett, H. L., *Biochem. J.*, 1913, vii, 441; *Proc. Roy. Soc. London, Series B*, 1914, lxxxvii, 300; *Biochem. J.*, 1914, viii, 204.

natural anthocyanins corresponding to the flavones are yet known, and the anthocyanins of Wheldale and Bassett may therefore have been very different in their properties from the anthocyanins prepared by Willstätter.

Geneticists who are working with cotton and have pure lines of color types at their disposal might turn to good account Perkin's investigations of the flavonol glucosides of several types of *Gossypium*.[15],[16] (Additional data have been contributed by Viehoever, Chernoff, and Johns.[17]) In cotton there is a series of Mendelian color types of which the basic work on the flavonols has been done, leaving only the anthocyanins to be worked out from the beginning.

We have already pointed out in another paper the value of Emerson's maize material for this problem. Although probably no better than cotton the maize is superior to *Antirrhinum* in containing flavonol rather than flavone glucosides, with the obvious advantage that the anthocyanins will probably prove to have been worked out, or at least to be very similar to those described by Willstätter.[8]

The maize series affords green types of different factorial composition which give, on crossing, a brown type. The brown type, obtained in the homozygous condition by inbreeding (self-pollination), will give simple Mendelian ratios when crossed with purple, the purple character being dominant in the first hybrid generation, and segregation in a 3:1 ratio taking place in the second generation. We have an example, then, of interacting factors, neither of which, alone, produces color, but which produce brown by interaction when both are present together. The symbols for these factors are B and Pl. The two different green types mentioned above contain B and Pl, respectively, each alone. A third factor is called A, which in the presence of B and Pl produces purple. With B alone the presence of A produces the type known as sun red, for the reason that the red color appears only if the plants are exposed to direct sunlight. With Pl alone, A produces a dilute purple color. A, then, is a factor for the production of some type

[15] Perkin, A. G., *J. Chem. Soc.*, 1899, lxxv, 825; 1916, cix, 145.

[16] Perkin, A. G., *J. Chem. Soc.*, 1909, xcv, 2181.

[17] Viehoever, A., Chernoff, L. H., and Johns, C. O., *J. Agric. Research*, 1918, xiii, 345.

of anthocyanin, the nature of which is determined by the other factors present. If A is present at all, the plant color in Emerson's material has always been purple or red. If the plant color is to be brown, A must be absent, but both B and Pl must be present. This interesting series of relations must have a discoverable chemical basis. To find such a basis has been the motive for the present paper, which is to be followed by others as the examination of the several genetical types proceeds.

Thus far we have worked mainly upon the brown type, since it seemed to provide the most tangible point of departure.

It might have seemed more logical to begin with one of the green varieties, of which there are three in Emerson's series, their formulas being aabbPlPl, aaBBplpl, and aabbplpl. The first two are the ones that give the brown type when crossed.

Using the same reasoning which has been advanced in the past, one might expect to find that these three green varieties would be characterized as follows: (a) one containing a chromogen, i.e. some colorless or relatively colorless mother substance from which flavonol or anthocyanin may be produced, but lacking the enzyme which accelerates the reaction, (b) a second variety containing the enzyme, but not the chromogen, and, (c) the ultimate Mendelian recessive, a variety containing neither chromogen nor enzyme. Lacking a clue to the nature of the hypothetical chromogen or mother substance of the pigment, however, and expecting only one of the three green genotypes to contain such a chromogen, it seemed best to work first with the brown type, upon the supposition that it would be found to contain a flavonol. This supposition was confirmed by the isolation and identification of quercetin, derived from an unidentified glucoside.

Preparation of Isoquercitrin.

The quercetin glucoside of the brown-husked maize was originally obtained in too small a quantity for thorough study by methods outlined in our former paper.[3] A much larger quantity was obtained subsequently by the following procedure. The ground husks were extracted for 2 or 3 weeks with 95 per cent alcohol, and the extract was evaporated under reduced pressure. The syrupy residue was taken up with boiling water and filtered by means of a hot water funnel. The filtrate was allowed

to cool and was then shaken with ether to remove free quercetin and other substances. The quercetin glucoside was then removed from the aqueous solution by repeated shakings with ethyl acetate. The ethyl acetate extracts were then evaporated in a distilling flask, and as tarry substances separated on the bottom of the flask they were eliminated by changing the flasks. As the process of concentration proceeded, this deposit became lighter in color, and was then filtered off and added to the residue obtained by complete distillation. The procedure affected a mechanical separation of the phlobaphene-like substances, which separated in a tarry condition and could easily be removed from the portions richer in the unidentified glucoside. The crude glucoside was purified by solution in successive small amounts of boiling water, followed by repeated crystallization from relatively large amounts of boiling water, aqueous pyridine, dilute acetic acid (1 per cent), and finally from boiling water. In the presence of contaminating substances, the quercetin glucoside was found to be easily soluble in boiling water. As it becomes purer the solubility decreases. Approximately 5 liters of boiling water were required to dissolve 11 gm. of air-dried glucoside, although this relation does not accurately represent the solubility ratio. A quantitative approximation of the glucosidal content of air-dried brown maize husks was determined by extracting 1,000 gm. with 95 per cent alcohol for 3 weeks. This extract was freed of alcohol by vacuum distillation and the residue taken up in boiling water as before. After filtering, cooling, and removing ether-soluble substances, the aqueous solution was shaken with ethyl acetate until the final extracts no longer gave a test for flavonol. Evaporation of acetic ether left a residue, which after initial separation from tarry substances and final purification by repeated crystallization from large volumes of boiling water, amounted to 4 gm. in the air-dried state. For carrying through much of the tedious work of extracting the glucoside our thanks are due to Mr. Paul Williams.

The corn glucoside, in its final state of purity, forms a felty mass of primrose-yellow needle-like plates, which are often branched as shown in Fig. 1. In our former paper[3] the color of this glucoside is reported as lemon-yellow and the melting point as 220–222°C. It is obvious that the small amount obtained for the previous investigation precluded the possibility of getting it pure and this

accounts for its deeper color and lower melting point. The pure glucoside melts at 220–222.5°C. An aqueous solution of this compound, using the filtrate from which most of the pigment had separated on cooling, gives with a few drops of ferric chloride, a pale olive-green, and with excess of the reagent, an intense olive-green color. Addition of sodium carbonate or dilute ammonia

Fig. 1 Isoquercitrin, of brown-husked maize crystallized from hot water (× 15).

to the pigment solution intensifies the original yellow color. Lead acetate added to the cold aqueous solution gives a yellow precipitate which becomes more voluminous on the addition of a trace of ammonia. When an aqueous solution is reduced with magnesium ribbon and hydrochloric acid a clear pale rose-red color is produced. Probably the spectral transmission curve

posi

affords as useful a criterion of the identity of a pigment as any that can be obtained. To facilitate future comparisons of unknown glucosides with the flavonol glucoside isolated from brown corn, the U. S. Bureau of Standards has very kindly prepared comparable spectral transmission curves (Fig. 2) of our maize glucoside and the quercetin prepared from it. In order to assure comparability the two pigments were dissolved in absolute alcohol and were made of the same molecular concentration (0.906 cg. of quercetin per liter; 1.392 cg. of isoquercitrin per liter, that is, solutions of м 300,000 concentration). The measurements in the visible part of the spectrum were made by Dr. M. K. Frehafer, and those in the ultra-violet by Mr. H. J. McNicholas, to whom we wish to express our thanks.

The spectral transmission of the glucosides of the flavone and flavonol derivatives will doubtless afford important evidence as to the position of attachment of the sugar residues, since the removal of a particular hydroxyl group by condensation with sugar might be expected to give a glucoside with approximately the same transmission as the non-glucoside with an H instead of an OH in the same position. Since the phenols and phenol acids formed as cleavage products of the flavones and flavonols (by fusion with alkali) indicate the position of the hydroxyl groups, it follows that the point of condensation in a glucoside would be indicated by a close similarity in optical properties between a glucoside of a given flavonol A, and a free flavonol B, containing one less OH group than A. Thus, if a monoglucoside of quercetin were closely similar in spectral transmission to kaempferol, it would indicate that the point of attachment of the sugar residue would be the particular hydroxyl which differentiates quercetin from kaempferol. The establishment of homologies between glucosides of the flavonol and anthocyanidin series is going to require the points of attachment to be known. It is therefore suggested that in future the measurement of spectral transmission be made a part of the routine examination of these glucosides, and that each curve be plotted against the free flavonol, in the same molecular concentration.

Identification of Isoquercitrin.

In our former paper[3] we stated that the glucoside of the brown maize was very similar to one isolated by Heyl[18] from the pollen of the ragweed, *Ambrosia artemisiafolia* L. A search of the literature showed that the latter was similar in many ways to the isoquercitrin described by Perkin[16] from cotton flowers. Heyl very kindly placed at our disposal the small sample of his compound which remained and Perkin likewise sent a liberal sample of authentic isoquercitrin. As a result of a careful comparison of the three preparations, we conclude that the corn glucoside is identical with isoquercitrin. We are not so certain about the identity of Heyl's compound with isoquercitrin, for the reason that the small amount available (90 mg.) was insufficient for complete identification. However, several points of identification have been made. All are monoglucosides. The products of hydrolysis in each instance are quercetin and glucose. All crystallize in the same form (see Figs. 1, 3, and 4). The melted glucoside in each case is a cherry-red oily liquid. Identical reactions are obtained when solutions of the three glucosides are reduced with magnesium and hydrochloric acid, and when they are treated with lead acetate, ferric chloride, sodium carbonate, and hydrochloric acid. Before we received samples of Perkin's isoquercitrin and Heyl's ragweed glucoside, a determination of the melting point of the corn glucoside led us to believe that all three compounds were different isomeric monoglucosides, since the melting point which we obtained for the corn glucoside differed from that of isoquercitrin, reported as 217–219°C.,[16] as well as from that of Heyl's glucoside[18] one sample of which melted at 224–226°C. and the other at 228–229°C. Since receiving these samples, however, we have found that the rapidity with which the heating is carried out in the melting point determination influences the melting point. When the compounds are placed in the bath at 200°C., and the heating is so regulated that there is a rise of 1° in 1 minute, the melting points are as follows: corn glucoside, 210–211°; Perkin's cotton isoquercitrin, 208.5–209.5°; Heyl's ragweed glucoside, 215.5–217.5°. Under similar circumstances, except that heating was at the rate of about 7.5° per minute, the melting

18 Heyl, F. W., *J. Am. Chem. Soc.*, 1919, xli, 1285.

points were 220–222.5°, 218–220°, and 224–226.5°, respectively. Heyl's compound mixed in an agate mortar with an equal amount of the corn glucoside did not show the depression in the melting point which would be expected if these two substances were different. The melting point of the mixture was slightly higher than that of the corn compound alone. Perkin's isoquercitrin

Fig 3. Heyl's ragweed quercetin monoglucoside, crystallized from hot water (× 450).

mixed with the corn glucoside changed the melting point of the latter only slightly, the difference being too small to be of any significance. Finally a mixture of all three glucosides was found to have a melting point identical with that of the corn glucoside. From the above results one is led to suspect that these three compounds will be found to be identical when larger amounts are available for examination.

One observation was made which may account for the difference in melting point between Heyl's glucoside and the other two. In crystallizing the three compounds under the same conditions, 0.034 gm. of air-dried pigment in each case dissolved in 5 cc. of boiling water, filtered hot and set aside to crystallize, we noticed that in each instance the crystalline precipitate was admixed with a small

FIG. 4. Perkin's cotton isoquercitrin, crystallized from hot water (× 450).

amount of droplets (amorphous spherules). Heyl's glucoside showed the least of this non-crystalline admixture and this may explain why his compound melted at a higher temperature than the other two. The amorphous spherules, according to this interpretation, represent some slight contamination, from which Heyl's compound is nearly free.

When deposited from aqueous solution, the corn glucoside contains water of crystallization, which is liberated at 160°C. The air-dried substance was, therefore, heated at this temperature to determine the loss of weight.

1.1554 gm. lost 0.0467 gm. H_2O. Found. H_2O 4.04.

In order to eliminate the error caused by the presence of hygroscopic moisture, the three following determinations were carried out using material which had been vacuum-dried at room temperature until it reached constant weight.

0.5229 gm. lost 0.0201 gm. H_2O. Found. H_2O 3.84.
1.1511 " " 0.0424 " " " " 3.68.
0.5980 " " 0.0239 " " " " 3.99.
$C_{21}H_{20}O_{12} \cdot H_2O$ requires: H_2O 3.73.
$C_{21}H_{20}O_{12} \cdot 2H_2O$ " " 7.20.

Analyses of the vacuum-dried (a) and anhydrous (b, c, d) glucosides are here given:

(a) 0.1302 gm.: 0.2491 gm. CO_2 and 0.0537 gm. H_2O. Found. C 52.18, H 4.62.

$C_{21}H_{20}O_{12} \cdot H_2O$ requires: C 52.26, H 4.59.

(b) 0.1229 gm.: 0.2441 gm. CO_2 and 0.0480 gm. H_2O. Found. C 54.17, H 4.38.

(c) 0.1043 gm.: 0.2057 gm. CO_2 and 0.0411 gm. H_2O. Found. C 53.79, H 4.45.

(d) 0.1824 gm.: 0.3623 gm. CO_2 and 0.0732 gm. H_2O. Found. C 54.16, H 4.49.

$C_{21}H_{20}O_{12}$ requires: C 54.29, H 4.34.

Isoquercitrin upon hydrolysis is resolved into glucose and quercetin in molecular proportions. Our sample of corn glucoside gave upon hydrolysis glucose and quercetin. For the quantitative determinations anhydrous material was used. The yield of quercetin accorded satisfactorily with that calculated from the equation:

$$C_{21}H_{20}O_{12} + H_2O = C_{15}H_{10}O_7 + C_6H_{12}O_6.$$

0.5424 gm.: 0.3521 gm. quercetin. Found. Quercetin 64.94.
0.5638 " 0.3650 " " " " 64.74.
$C_{21}H_{20}O_{12}$ requires: Quercetin 65.08.

The above determinations were carried out by hydrolyzing the samples (oven-dried at 160°C.) by boiling with 100 cc. of approx-

imately 4 per cent H_2SO_4 for 1 hour and then placing on a boiling water bath for another hour. The mixture was cooled over night, filtered, and the residue thoroughly washed with cold water and dried to constant weight at 130°C. The acid filtrates gave positive rotation indicating the presence of a dextro-rotatory sugar. Excess of sodium acetate and phenylhydrazine was then added and the solution heated on a boiling water bath until a yellow crystalline osazone separated. This was recrystallized from water containing 5 per cent pyridine, then dissolved in a small amount of pyridine to which were then added hot alcohol and a little hot water. The characteristic glucosazone, melting at 205–205.5°, crystallized out.

The quercetin obtained from the glucoside by hydrolysis was identified by all the usual tests and by combustions of both hydrated (a) and anhydrous (b, dried at 130°C.) samples. A determination of the water of crystallization in a sample of quercetin exposed to the air for 1 month and the combustion results are as follows:

0.6220 gm. lost, at 130°C., 0.0656 gm. H_2O.   Found. H_2O 10.54.
$C_{15}H_{10}O_7.2H_2O$ requires: H_2O 10.65.

(a) 0.1652 gm.: 0.3229 gm. CO_2 and 0.0630 gm. H_2O.   Found.   C 53.30, H 4.27.
$C_{15}H_{10}O_7.2H_2O$ requires: C 53.24, H 4.18.

(b) 0.1890 gm.: 0.4121 gm. CO_2 and 0.0580 gm. H_2O.   Found.   C 59.46, H 3.43.
$C_{15}H_{10}O_7$ requires: C 59.59, H 3.34.

Combustions of acetyl quercetin, dried at 160°C., gave the following figures:

0.1301 gm. gave 0.2794 gm. CO_2 and 0.0480 gm. H_2O.   Found.   C 58.57, H 4.13.
0.1277 gm. gave 0.2744 gm. CO_2 and 0.0440 gm. H_2O.   Found.   C 58.59, H 3.86.
$C_{15}H_5O_7(C_2H_3O)_5$ requires: C 58.57, H 3.93.

The hydrolysis of the penta-acetylquercetin carried out in glacial acetic acid by means of hydrochloric acid gave the following results:

0.6408 gm.: 0.3753 gm. quercetin (dried at 130°C.).   Found.   Quercetin 58.65.
1.0578 gm.: 0.6204 gm. quercetin (dried at 130°C.)   Found.   Quercetin 58.56.
$C_{15}H_5O_7(C_2H_3O)_5$ requires: Quercetin 58.98.

SUMMARY AND CONCLUSIONS.

1. A series of color types in maize involving pigments of the flavonol and anthocyanidin groups, has been genetically analyzed by Emerson. The known genetic constitution of these color types makes it very desirable to conduct parallel chemical investigation with them, in order to understand the operation of the Mendelian factors involved.

2. As a beginning in this work, we have isolated a flavonol glucoside from brown-husked maize, one member of Emerson's series and the lowest one showing the presence of a pigment of the groups under consideration. This glucoside is found to be the same as Perkin's isoquercitrin, originally isolated from cotton, and probably the same as an unnamed glucoside isolated by Heyl from pollen of ragweed. It is a monoglucoside yielding only glucose and quercetin on hydrolysis. The large yield of this glucoside obtained from corn husks made it possible to add to the characterization of isoquercitrin by Perkin who had only a limited amount at his disposal. As a contribution to the more accurate identification of such pigments, the spectral transmission has been determined by McNicholas and Frehafer of the U. S. Bureau of Standards. Their data are published in Fig. 2.

3. It is suggested that spectrophotometric and spectrographic comparison of the pigments of the flavones, flavonols, and anthocyanidins may possibly afford the best evidence as to the points of attachment of the sugar residues in the glucosides.

CHART FOR THE CONVERSION OF COLORIMETRIC READINGS INTO HYDROGEN ION CONCENTRATION.

By J. F. McCLENDON.

(From the Laboratory of Physiological Chemistry, University of Minnesota Medical School, Minneapolis.)

The most popular method of determining hydrogen ion concentration by the use of indicators has been the method of making buffer mixtures of known hydrogen ion concentration and adding indicators to them; at the same time adding the same quantity of indicator to the same volume of the unknown mixture and noting the buffer mixture that matched the unknown in color. This method necessitated knowing two things: one was the hydrogen ion concentrations of the buffer mixtures and the other was the difference in salt error between the buffer mixture and the unknown. In the practical working of the method other difficulties arose: (1) errors due to concentration of the indicator, since the same quantity of indicator had to be added to the buffer mixture and to the unknown, and (2) errors due to impurities in the indicator. These errors were difficult to avoid when the buffer mixture was sealed up and kept for a long time after the indicator had been added. If, however, the indicator is added to the standard at the same time it is added to the unknown, such errors can easily be avoided since the same sample of indicator can be used in adding portions to the unknown and to standard solutions. Perhaps on account of the above difficulties a number of persons have recently been using methods based on the percentage of the indicator that is in the so called dissociated condition.

We do not wish here to go into the theory of the color production, but take the simple hypothesis of Ostwald that the free indicator is undissociated and the salt of the indicator is largely, and under certain conditions, 100 per cent dissociated. Perhaps the simplest application of this method is the use of indicators which are

647

colorless in the undissociated condition, such as phenolphthalein, and compare the colors in a Duboscq colorimeter as follows: Equal volumes of unknown solution and of distilled water that has been made alkaline are taken, and to each of these the same quantity of indicator is added. In the alkaline-distilled-water-solution it is presumed that 100 per cent of the indicator is dissociated or in the red condition. This is tested by adding more alkali until the indicator solution does not become any redder. The unknown solution with indicator is placed in the left-hand cup of the colorimeter and this cup set at some known point, such as 10 mm., between the plunger and the bottom of the cup. The alkaline solution of the indicator is added to the right-hand cup and the plunger is raised and lowered until a color match is obtained. The reading on the right millimeter scale, if multiplied by 10, will give the percentage of the indicator that is dissociated in the left-hand cup. It remains to interpret this in terms of hydrogen ion concentration, which is done by the following formula:

$$\alpha = \frac{K}{K + [H^+]}$$

α is the degree of dissociation, K is the dissociation constant, and $[H^+]$ is the concentration of hydrogen ions.

Such calculations, however, although they are done easily by many workers, are only accomplished with difficulty by others, and often the liability for error is proportional to the difficulty. It therefore seems desirable to use the graphic method of working out this formula. Clark, in his book on determination of hydrogen ions, has drawn the graphs for a number of indicators, showing the relation of hydrogen ion concentration to percentage dissociation. These graphs are curves which are not easily drawn by everyone, and, therefore, it seemed desirable to change the graph paper in such a way that the curves would become straight lines. This cannot be done with any of the ordinary graph papers in use, and when it is done the different regions of the graph have different ratios of numerical value to actual dimensions, and some slight error may arise in interpolation between the lines on the graph. Such errors, however, should be small and, therefore, it seemed desirable to publish the accompanying graph with tentative values for a number of indicators. The diagonals

represent the same functions as the curves in the charts in Clark's book and therefore should be easily understood by anyone familiar with hydrogen ion determinations.[1]

We add phenolphthalein, for instance, to the unknown solution coming within its range placed in the left-hand cup of the Duboscq colorimeter and to alkaline water in the right-hand cup. If the left-hand cup is set at 10 mm. and the right-hand cup adjusted so as to obtain a color match, the reading on the right-hand scale multiplied by 10 will give the percentage dissociation of the indicator in the unknown solution. This percentage dissociation is then transferred to the chart and its coordinate following to diagonal for phenolphthalein. The point of intersection will give on the abscissa the pH value (or logarithm of the reciprocal of the hydrogen ion concentration). The same principle can be used with thymolphthalein, α-naphtholphthalein, and related indicators. In case of the sulfonic acid compounds of these indicators the Duboscq colorimeter, as ordinarily used, will read with difficulty, owing to the fact (with phenol red, for instance) that the unknown solution in the left-hand cup will show orange, which is a mixture of red and yellow, whereas the right-hand cup will show only different intensities of red as the cup is moved up or down. The eye cannot evaluate the red in the orange without the use of a color screen which would absorb the yellow. The color screen would be placed between the eye and the eyepiece of the colorimeter.

Another method would be to use a color screen absorbing the red or to use monochromatic light in illuminating the apparatus. Suppose we use sodium light. This would pass through the yellow solution and yet it would be absorbed by the red and hence the red alone or the red element in the orange would appear dark and a matching of the two sides would be a matching of the intensity of monochromatic light.

[1] See Clark, W. M., The determination of hydrogen ions, Baltimore, 1920. The equation on which the chart is based is No. 7, p. 20:

$$pH = \log \frac{1}{[H^+]} = \log \frac{1}{K} + \log \frac{\alpha}{1 - \alpha}.$$

Values of $\log \frac{\alpha}{1 - \alpha}$ are given on p. 306.

A simple method of matching these sulfonic acid dyes, such as phenol red, has been used by Barnett and Barnett[2] and fully described by them. The principle of this is a rectangular trough of glass, divided diagonally by a vertical sheet of glass into two compartments. The equal volumes, say 100 cc. of unknown and of two distilled water solutions, are carefully measured out and the same quantity of indicator added to each. One of these distilled water solutions is made acid until the indicator is all in the yellow form and the other made alkaline until the indicator is all in the red form and the yellow solution is added to one compartment and the red to the other. If we then look through the trough in the horizontal direction, the color shades from yellow at one end through various shades of orange to red at the other end. In another rectangular trough, made the same thickness as this one, the unknown is placed. The unknown is then moved back and forth above the former and matched with some portion of the long trough. This point of matching is then measured in percentage of the total length, beginning at the yellow end; that is to say, the percentage red is determined in this way. With this value of percentage red (percentage dissociation) we may use Chart 1 as we did with phenolphthalein.

Victor Myers[3] has demonstrated a double-wedged colorimeter built on the same principles as this simple trough and the values obtained with it may be converted into pH values by Chart 1.

Another apparatus that has been used is a Duboseq colorimeter with two cups on the right-hand side, one movable and working inside the other. This has been described with detailed instructions as to its use.[4] One of these instruments was made under the direction of E. P. Lyon and used considerably, the values being interpreted by means of Chart 1 and compared with values obtained by the use of buffer solutions. The method is simply the placing of the unknown in the left-hand cup (colored with phenol red, for instance) and taking the red solution out of Barnett's apparatus and placing it in the inner cup and the yellow solution out of Barnett's apparatus and placing it in the outer cup. The outer

[2] Barnett, G. D., and Barnett, C. W., *Proc. Soc. Exp. Biol. and Med.*, 1920–21, xviii, 127.

[3] Myers, V. C., *J. Biol. Chem.*, 1922, l, p. xxii.

[4] Gillespie, L. J., *J. Bact.*, 1921, vi, 399.

CHART 1.

cup and the plunger are fixed and only the inner cup is movable. The scale on the right-hand side measures in millimeters, but applies to the movement of the inner cup only. The outer cup is fixed the same distance from the plunger as the left-hand cup, namely 10 mm. in this case. The unknown will show an orange color. By moving the inner cup up and down an exact color match will be obtained. When the cup moves upward the red is decreased and the yellow increased and when it is moved downward the red is increased and the yellow decreased.

The chief value of Chart 1 probably rests in the ease with which new data may be added to it. For instance, let us suppose that we have an indicator that is not on the chart. We first determine its dissociation constant (K). If we take the logarithm of the reciprocal of the dissociation constant and find its numerical value on the pH scale and make a mark where it crosses the 50 per cent coordinate, and draw through this point of intersection a diagonal line parallel to the diagonals on the chart, we will have a "curve" for converting values obtained with this indicator into pH values as has been described. Some samples of indicator may show dissociation constants different from those of other samples and new diagonal lines for these new samples may be made in this same manner. Also the presence of neutral salts may change the dissociation constant of an indicator and in this way a new diagonal for a different salt concentration may be drawn. It is not even necessary to know the dissociation constant. If we can determine the dissociation at any pH value and fix this point on the chart and draw a diagonal through this point parallel to the diagonals, we have the values for the new indicator or for any indicator under new conditions.

The different regions of the chart are not of the same sensitiveness so far as the detecting of the color change is concerned. The eye is subject to Weber's law, that is to say, the eye detects a percentage change in a color, and the absolute change it can detect is less the greater the intensity of the color. For this reason the portion of the chart showing less than 50 per cent dissociation is more sensitive than that showing more than 50 per cent. In case of methyl red, however, the reciprocal of the dissociation was used in drawing its curve in order to obtain the same slope of the curve as in the case of acid dyes. This will lead to no error

in practical application since the same conventions in the acid
and alkaline solutions of the dye will be used, but the portion of
the chart that is marked for more than 50 per cent dissociation
would be the most sensitive region for methyl red.

Note Added to Proof.—According to Michaelis and Gyemant,[5] the curve
for phenolphthalein is not quite a straight line as in the chart. Their
figures for 100 α and pH are as follows: 10, 8.95; 20, 9.20; 30, 9.35; 40, 9.50;
50, 9.70; 60, 9.90; 70, 10.10; 80, 10.30. They also add the following useful
indicators with the pH at $\alpha = 50$ per cent; 2,6-dinitrophenol, 3.69; 2,4-
dinitrophenol, 4.06; *p*-nitrophenol, 7.18; *m*-nitrophenol, 8.35. They made
these determinations at 18° and they found that rise in temperature moves
the figures to the acid side; that is to say, the values for pH decrease
slightly.

[5] Michaelis, L., and Gyemant, A., *Biochem. Z.*, 1920, cix, 165.

STUDIES ON ENZYME ACTION.

XXI. BANANA GEL AND BANANA SUCRASE.

By K. GEORGE FALK and GRACE McGUIRE.

(From the Harriman Research Laboratory, The Roosevelt Hospital, New York.)

INTRODUCTION.

Some of the properties of the sucrolytic enzyme obtained from bananas were described in a previous paper.[1] The conditions for action, the occurrence of soluble and insoluble forms, and a transformation of the former into the latter, were discussed. In a subsequent paper,[2] the properties of banana gels, their formation, and conditions of relative stability, were described without, however, considering the accompanying enzyme actions.

In this paper the properties of the banana gels will be considered more fully, especially in connection with the sucrase actions of the preparations. The topics studied may be grouped as follows: (a) Relation between gel formation and sucrase content of gel with sodium chloride extracts of banana pulp; (b) similar studies comparing sodium chloride, sodium nitrate, sodium sulfate, and magnesium sulfate extracts; (c) sucrase contents of gel and solution, with gel prepared by the action of pancreatin on banana pulp extract; and (d) separation of gel and sucrase substances by various treatments.

EXPERIMENTAL METHODS AND RESULTS.

The method of obtaining banana extracts was similar to that described previously[1] and is briefly as follows: Ripe bananas were peeled, the pulp was passed through a food chopper, the finest cutter being used, mixed thoroughly with the extracting solu-

[1] Falk, K. G., and McGuire, G., *J. Gen. Physiol.*, 1920–21, iii, 595.

[2] McGuire, G., and Falk, K. G., *J. Gen. Physiol.*, 1921–22, iv, 437.

tion, and after standing a definite length of time (45 minutes as a rule), poured on a large folded filter on ribbed funnels. Filtration proceeded slowly and the filtrate was collected for about 18 hours. Longer time and filtration through fresh filter papers gave very little additional filtrate. Toluene was added to the pulp mixture when it was put on the filter and to the filtrate. It was not added to and mixed with the pulp before filtration because of its possible precipitating, coagulating, or other action. The activity of preparations was tested in a manner similar to that already described. The enzyme preparation was incubated at 37.5° for 3 to 24 hours, diluted, and the reducing substances were determined by the reduction of Fehling's solution.[3]

The results obtained in extracting the same quantity of pulp with different quantities and concentrations of sodium chloride solution as contrasted with water extraction and with no added liquid, are given in Table I.

The results with the sodium chloride extractions given in Table I were obtained with two different batches of bananas, Series A and B. The results with the different batches are not strictly comparable because of the variations to be found in the compositions, etc., of different bananas, but the general relations are always the same. The conclusions to be drawn from these results are as follows:

1. Gel formation decreased with increase in sodium chloride concentration of the extracting liquids (confirming former results).

2. The sucrase actions of the extracts (as shown by the activities of the gels) increased with the presence of sodium chloride in the extracting liquids. A very large increase in the amount of extracting liquid (sodium chloride solution) showed a decrease in sucrase concentration although an increase in total sucrase extracted.

3. The volumes of liquid retained by the pulp varied with the different batches. With a certain addition of extracting liquid, water or solution derived from the pulp was given up and appeared in the filtrate; with a greater addition, water or solution was retained by the pulp. The amount of liquid retained by the pulp in any one series appeared to be independent of the presence or

[3] Sherman, H. C., Kendall, E. C., and Clark, E. D., *J. Am. Chem. Soc.*, 1910, xxxii, 1083.

TABLE I.

Gel Formations and Sucrase Contents of Gels Obtained with Sodium Chloride Extracts of Banana Pulp.

Experiment No.	Extracting liquid.	Molarity (NaCl) of mixture, assuming pulp to be 25 per cent solids.	Filtrate in 20 hrs.	Volume after dialysis.	25 cc. filtrate dialyzed against tap water for 48 hrs.			
					Gel formation upon dialysis.	Filtrate from gels after dialysis.	Sucrase actions (4 hrs.) of total gels.	
						cc. (In 5 days.)	*mg.* Cu_2O	
			cc.	*cc.*				
	Series A. Portions of 600 gm. pulp.							
1	None.	No added liquid.	45	55	Excellent.	18	7,000	
2	75 cc. M NaCl.	0.14	100	52	"	22	15,000	
3	150 " " "	0.25	120	48	"	21	17,000	
4	600 " " "	0.57	190	35	Less firm.	23	Gel unsatisfactory for test.	
5	900 " " "	0.67	230	32	Very soft gel.	27	Gel unsatisfactory for test.	
	Series B. Portions of 380 gm. pulp.					(In 1 to 1½ hrs.)		
6	95 cc. H_2O.	Water added.	120	43	Good.	1.0	Trace.	
7	95 " M NaCl.	0.25	126	43	"	1.0	6,000	
8	380 " H_2O.	Water added.	206	33	Fair.	10	Trace.	
9	380 " M NaCl.	0.57	180	33	"	5	4,950	

absence of sodium chloride in the extracting liquid (also shown by experiments not quoted in the table).

4. On dialysis against tap water of the pulp filtrates, the smaller the amounts of added extracting liquids, the greater the relative increase in volume. The gel-forming property of the pulp filtrates on dialysis was greater, the smaller the amounts of added extracting liquids.

The presence of calcium salts in the sodium chloride in experiments similar to those of Table I, Series B, with the same batch of bananas showed gel formation with the undialyzed extract with a smaller concentration of added calcium salt (and suitable alkali) than in the extraction with pure sodium chloride solution, while the gel formation upon dialysis against tap water and the solubility of the sucrase were the same in the two cases.

The filtrates from the gels shown in Table I in almost all the experiments contained no sucrose. In a few isolated experiments, on prolonged incubation with sucrose, a trace of activity was observed.

The results obtained in the extraction of gel-forming substance and of sucrase substance by solutions of various salts of different concentrations including sodium chloride, sodium nitrate, sodium sulfate, and magnesium sulfate are given in Table II. A 1:2 ratio of cc. of extracting liquid to gm. of pulp was used in each case. Each experiment of Series A, B, C, and D, was carried out with one batch of bananas and different concentrations of a salt, while Series E compared a number of different salts at their most satisfactory concentrations for sucrase extraction on the pulp from the same batch of bananas. The extracts were dialyzed against tap water for 18 hours and then tested for sucrase action and for gel formation with calcium chloride solution and ammonium hydroxide to bring the mixtures to pH 7.0. With the exception of 1.2 M magnesium sulfate extract, the gel was just beginning to form, or had formed only to a very small extent after 18 hours dialysis, and did not prevent the formation of a comparative gelling series upon the addition of calcium salt and alkali. The extracting solutions and filtrates were all at pH 5.0.

The conclusions which may be drawn from the results given in Table II are as follows:

1. Increase in concentration of the salt decreased the amount of gel-forming substance extracted in the sodium chloride and sodium nitrate series, and in the magnesium sulfate series in the more concentrated solutions. Very little difference in this property was observed in the sodium sulfate series, possibly because of the batch of bananas used as shown by the gel from the water extract.

2. Salt solutions extracted more sucrase than did the water alone. (a) With sodium chloride, increase in the concentration of salt increased the amount of sucrase extracted with a possible maximum concentration between 1 and 2 M. (b) With sodium nitrate, a maximum sucrase extraction was obtained at about 0.24 M, higher salt concentrations not increasing the amount of sucrase. (c) With sodium sulfate, a similar maximum was obtained at 0.12 M. (d) With magnesium sulfate, a similar maximum was obtained at 0.24 M. (e) With different salts at their maximum sucrase extraction actions, the concentrations of sucrase extracted from the same pulp differed only to minor extents.

3. No regularity exists between the amounts of the filtrates, or amounts of liquid held back or retained by the pulp, and the concentration of salt, except for the case of sodium nitrate, where more liquid was held back by the pulp with increase in concentration of the sodium nitrate.

4. On dialysis against tap water in collodion bags for 18 hours, the increases in the volumes of all the filtrates were of the same order of magnitude and independent of the salts and their concentrations in the filtrates.

As in the results shown in Table I, the filtrates from the gels showed no sucrase activity.

If the banana pulp filtrates as originally obtained were boiled for a few minutes, no gels were obtained either on dialysis or on treatment with calcium salts in alkaline solutions. Boiling also destroyed the sucrase actions of the extracts.

The experimental data relative to the formation of gel from banana pulp extracts by the action of a pancreatin preparation (U. S. P. VIII Revision) on incubation at 37° at pH 5.0, and the distribution of the sucrase between the gel formed in this way and the liquid are given in Table III. These experiments were planned originally for an entirely different purpose. It was

TABLE II.

Gel Formations and Sucrase Contents of Gels of Extracts of Banana Pulp with Solutions of Various Salts.

Experiment No.	Extracting liquid.	Molarity of mixture, assuming pulp to be 25 per cent solids.	Filtrate.		50 cc. pulp filtrate dialyzed 18 hrs. against tap water.		
					Volume after dialysis.	Gel formation CaCl$_2$ + NH$_4$OH.	Activity of dialyzed mixture tested 4 hrs. Calculated to cc. undialyzed extract.
			hrs.	cc.	cc.		mg. Cu$_2$O
			Series A.				
1	300 cc. H$_2$O.	Water added.	7	100	100	Good.	13
2	300 " 0.3 M NaCl.	0.12	7	170	90	Decreasing →	61
3	300 " 0.6 "	0.24	7	210	90		155
4	300 " 1.5 "	0.60	6	135	90		215
5	300 " 3.0 "	1.20	6	260	90	None.	272
			Series B.				
6	450 cc. H$_2$O.	Water added.	6	350	85	Good.	64
7	450 " 0.6 M NaNO$_3$.	0.24	5	310	90	Decreasing →	534
8	450 " 1.5 "	0.60	5	270	90		576
9	450 " 3.0 "	1.20	6	180	90		524
10	450 " 6.0 "	2.40	6	130	90	Very poor.	544
			Series C.				
11	450 cc. H$_2$O.	Water added.	2	200		Soft.	12
12	450 " 0.3 M Na$_2$SO$_4$.	0.12	5	170	95	"	270
13	450 " 0.6 "	0.24	5	340	100	"	260
14	450 " 1.32 "	0.53	5	180	95		240

Series D.

		Water added.					
15	450 cc. H₂O.	Water added.	5	460	85	Fair.	29
16	450 " 0.3 M MgSO₄.	0.12	5	620	75	"	377
17	450 " 0.6 " "	0.24	5	500	85	"	491
18	450 " 1.5 " "	0.60	5	660	100	Poor.	491
19	450 " 3.0 " "	1.20	5	500	130		494
20	450 " 5.0 " NaCl.	2.00	5	345	90		490

Series E.

21	265 cc. H₂O.	Water added.	3	240	80	Not tested.	28
21	450 " "	"	3½	380	80		25
22	450 " 3.0 M NaCl.	1.20	3	190	80	Not tested.	210
22	450 " 3.0 "	1.20	2	185	80		230
23	450 " 0.6 " NaNO₃.	0.24	3	170	75	Not tested.	251
23	450 " 0.6 " "	0.24	2	280			
24	450 " 0.6 " Na₂SO₄.	0.24	3	240	85	Not tested.	290
24	450 " 0.6 " "	0.24	2½	325	85		(180)
25	450 " 0.6 " MgSO₄.	0.24	3½	470	75	Not tested.	245
25	450 " 0.6 " "	0.24	2	270	70		250

thought that the action of the pancreatin enzymes might result in the decomposition of the substances which formed gels with calcium salts and in this way possibly throw light on the chemical nature of these substances. In place of destroying the gelforming substances in the banana extracts, the pancreatin itself caused the formation of a gel. The conclusions from the results shown in Table III are as follows:

1. The filtrates from the gels showed marked sucrase activities. Longer times of incubation were found to diminish these activities, perhaps because of the higher temperature of incubation (results not shown in the table).

2. The filtrates from the gels on treatment with calcium salts at pH 7.0 or more alkaline conditions did not form any gels but only cloudy solutions with slight precipitates.

3. Gel formation occurred at pH 5.0 on incubation of the banana pulp filtrates with pancreatin at 37°. Different salt extracts formed gels in different lengths of time. Sodium sulfate extracts formed gel more slowly than did the aqueous extracts or the sodium chloride or sodium nitrate extracts. None of the extracts was dialyzed before incubation.

4. If the banana pulp filtrates as originally obtained were boiled for a few minutes, subsequent incubation with pancreatin did not result in the formation of gels.

3 days treatment of a sodium chloride extract at 5° with pancreatin did not result in the formation of a gel. Boiled pancreatin solution incubated with banana pulp filtrates at 37° did not form gels.

The pancreatin preparation showed no sucrase action.

No attempt was made to measure the sucrase content of the pancreatin gel because of sucrase present in the bathing liquid.

An experiment was carried out with a sodium chloride (0.25 M) extraction of banana pulp at pH 3.5 (hydrochloric acid added to bring about this reaction). The filtrate on standing at room temperature showed the separation of a gel after 6 days. This gel gave marked sucrase action while the filtrate from it gave none.

The results obtained in the conversion of the "insoluble" form of sucrase (in the calcium gel) into a soluble form are given in Table IV.

TABLE III.

Gel Formation and Sucrase Contents of Gel Filtrates from Banana Pulp Extracts Treated with Pancreatin.

Experiment No.	Banana pulp	Extracting liquid	Molarity of mixture, assuming pulp to be 25 per cent solids.	Volume of filtrates after 18 hrs.	50 cc. pulp filtrate to 0.1 gm. pancreatin at 37°.		Clear filtrates from pancreatin gels dialyzed 48 hrs. against tap water.		Sucrase action of dialyzed filtrate from pancreatin gel, calculated to 1 cc. undialyzed extract
					Time.	Gel formation.	Gel formation on dialysis.	Original volume 50 cc. increase after dialysis.	
	gm.			cc.	hrs.			cc.	mg. Cu_2O
1	1,545	480 cc. M NaCl	0.30	880	18 / 26	No gel. / Gel.	No gel; liquid cloudy.	76	199
2	1,100	350 cc. M NaNO$_3$.	0.25	500	18	"	No gel; liquid cloudy.	92	315
3	1,000	265 cc. 0.5 M Na$_2$SO$_4$.	0.12	600	26 / 48	No gel. / Gel.	" " "	101	433
4	1,000	265 cc. M Na$_2$SO$_4$.	0.25	490	72	No gel. Very cloudy.	No gel; liquid cloudy.	87	113

TABLE IV.

Distribution of Sucrase in Gels and Filtrates after Various Treatments.

Experiment No	Salt used and treatment of extract.	Part tested.	Activity calculated to 1 cc. undialyzed extract.
	Series I. Sodium sulfate extract.		
			mg. Cu$_2$O
1	Gel mixture formed by dialyzing 27 hrs. against tap water.	Mixture. Filtrate. Gel.	510 0 340
2	27 hr. tap water gel from Experiment 1, dialyzed 4 days against distilled water. Filtered through paper.	Filtrate.	260
3	Unfiltered distilled water mixture from Experiment 2, centrifuged in Sharples supercentrifuge at 35,000 R.P.M. for 7 min. Filtered through paper.	Filtrate.	370
4	Gel mixture formed by dialyzing 48 hrs. against tap water.	Mixture.	500
5	48 hr. tap water gel from Experiment 4, dialyzed 4 days against distilled water. Filtered through paper.	Filtrate.	200
	Series II. Magnesium sulfate extract.		
6	Gel mixture formed by dialyzing 27 hrs. against tap water.	Mixture. Filtrate. Gel.	350 0 340
7	27 hr. tap water gel mixture from Experiment 6, dialyzed 4 days against distilled water. Filtered through paper.	Filtrate.	290

The conclusions from the results in Table IV are as follows:

1. Tap water or calcium gel upon filtration through paper gave a water-clear, colorless, inactive filtrate and an active light brown gel.

2. Tap water or calcium gel dialyzed against distilled water lost its gel-like form and upon filtration through paper gave a slightly cloudy, active filtrate.

3. Centrifuging the unfiltered active liquid from Experiment 2 at 35,000 R.P.M. for 7 minutes threw out a very small amount of solid matter and upon filtration through paper gave a more active filtrate. Presumably, the fine particles thrown out in the Sharples supercentrifuge tended to clog the paper filter and hold back some of the activity present in colloidal form.

The results in the table refer to several of the experiments with sodium sulfate and magnesium sulfate extracts. Similar results were obtained with sodium chloride and sodium nitrate extracts. Because of the small activity of the aqueous extracts, the gels formed from these were not tested in this way, although they were found to be destroyed by similar dialysis.

Numerous other attempts to convert the insoluble into the soluble form were unsuccessful.

<p style="text-align:center">DISCUSSION.</p>

The work described in this paper was carried out with the primary intention of studying the properties of the enzyme sucrase in so far as these involve the possibility of separation from insoluble material, and the relation of the soluble to the insoluble enzyme. In the course of this investigation it was necessary to enter into the question of gel formation, etc., to a certain extent.

The first important conclusion is that the gel-forming property and the sucrase property of the banana are due to different substances. This is shown in the first place by the greater solubility of the gel-forming substance in water and of the sucrase substance in salt solution. Both properties are, however, destroyed by boiling their solutions.

The gel formation was brought about by dialysis against tap water (of a certain calcium salt content and alkalinity) by treatment with calcium salt and alkali, and by incubation with a pancreatin preparation. In order to study the sucrase actions, the second method of gel formation could not be used, because the alkali inactivated the sucrase to a considerable extent. In the first method, presumably the alkali penetrated the collodion bag very slowly, the gel forming first along the inner wall of the bag, in

this way causing only slight inactivation of the enzyme. The calcium gel could be destroyed without simultaneous inactivation of the accompanying sucrase by dialysis against distilled water. Attempts to remove the calcium by precipitation would involve the use of alkali, and were, therefore, ruled out.

The calcium gels contained all the sucrase present originally, the pancreatin gels only a part or none. Although the properties of the calcium and pancreatin gels may be different, they involve the same substance or substances, since the filtrate from each gave no gel by the other treatment. The formation of a gel by the action of pancreatin on banana extract is of special interest in view of the work of Willstätter and Racke[4] who treated yeast cells by different methods, including the actions of enzymes, to bring the yeast sucrase into solution. It may be pointed out that the gel formation in the present instance appeared to be connected with the presence of an amylolytic or similar enzyme and not with the proteolytic action, since the use of papain in place of pancreatin did not produce a gel.

The molecular aggregates or micellæ which form the gel include within themselves the whole of the sucrase present when the gel was formed by the addition of calcium salt and suitable alkalinity, but only a portion or none of the sucrase when the gel was formed by the action of pancreatin. The properties of the gels should be explainable upon the basis of the principles developed by Loeb, Proctor, and Wilson.[5] A practical difficulty in the present connection lies in the fact that the gels obtained did not consist of one substance, or one group of substances. In the neighborhood of 25 per cent of the solid matter of the gel was protein in character, while the remainder consisted essentially of complex carbohydrate material. Also, in view of the problem studied, the hydrogen ion concentrations were not varied over wide ranges because of the possible inactivating effects on the sucrase. At the same time, some of the results are of interest in the general study of gel behavior.

The retention of water by the gels on filtering through paper (as measured by the volumes of the filtrates) may be taken as a rough measure of the swelling properties of the gels. Because

[4] *Cf.* Willstatter, R., and Racke, F., *Ann. Chem.*, 1922, cdxxvii, 111.

[5] Summarized by Loeb, J., Proteins and the theory of colloidal behavior, New York, 1922.

of the unsatisfactory nature and the complexity of the substances present in the original banana pulp, the volumes of the filtrates obtained in its extraction with different concentrations of various salts gave in the main irregular results and are not useful in the present connection. These filtrates (at pH 5.0) on dialysis in collodion bags against tap water (pH 7.0 with a certain calcium salt content) showed very nearly the same increases in volumes independent of the specific salt and its concentration which was used in the extraction. The volumes of the gels formed depended upon the concentration of the gel-forming substance extracted from the banana pulp by the solution in question. The results showed that the sucrase substance as such played no part in the swelling. Unquestionably, if comparable conditions of dialysis were used, it would be found that the Donnan equilibrium played an important part in the retention of water by the gels,[6] although as Loeb pointed out, other forces may also be involved in such an action.[7] This question, however, was not studied further in the present connection.

The conditions for the change of the sucrase from the insoluble to the soluble state and *vice versa* are of main interest in the present connection. Sucrase from various sources has been found, as a rule, to be soluble in water. An exception, however, was described some years ago by Vinson,[8] who showed that the sucrase of the green date was insoluble and that of the ripe date soluble in water.

These results show the reciprocal changes of the soluble and insoluble forms of sucrase by simple treatments and involving in the main the substances present in the material from which the enzyme was originally obtained. Similar changes have been shown to occur when certain insoluble colloids such as aluminum hydroxide, charcoal, fullers' earth, etc., were added to soluble sucrase preparations and found to remove the sucrase from solution completely under certain conditions, and upon the addition of certain substances (phosphate or citrate mixtures containing sucrase, etc.) to return the sucrase to the solution.[9] In a sense

[6] Loeb,[5] Chapter XI.

[7] Loeb,[5] p. 194.

[8] Vinson, A. E., *J. Am. Chem. Soc.*, 1908, xxx, 1005.

[9] Griffin, E. G., and Nelson, J. M., *J. Am. Chem. Soc.*, 1916, xxxviii, 722. Nelson, J. M., and Hitchcock, D. I., *J. Am. Chem. Soc.*, 1921, xliii, 1956. Willstätter, R., and Kuhn, R., *Z. physiol. Chem.*, 1921, cxvi, 53. And others.

these changes may be looked upon as changes in solubility of definite substances (whose exact chemical nature is unknown at present) brought about by combination with substances whose chemical natures in some instances are known.

This discussion leads to the conclusion that a definite enzyme property is not a soluble molecule or an insoluble molecule as such. The evidence indicates that, in the present case, the sucrase property on the one hand may be connected with, or a part of, a water-soluble molecule (or soluble in solutions containing various salts) and on the other hand, as the result of comparatively simple chemical treatments, changed so that it is connected with, or a part of, an insoluble compound. If the hydrogen ion concentrations for optimum actions are the same for soluble and insoluble enzyme preparations (either existing naturally, or prepared as such *in vitro*), then the conclusion is permissible that the enzymically active parts are identical. The hydrogen ion concentration optima are the properties most readily measured quantitatively and are therefore most frequently used, but other chemical properties, such as actions upon different simple substrates, are desirable. The simplest chemical interpretation is that the enzyme action is due to certain groupings present at one time with soluble material, at another time with insoluble material.

The relation between soluble and insoluble sucrase has been developed here. Sucrase is especially suitable for these studies because of its comparative stability. The conclusions are, however, applicable to other enzymes. For example, it has been shown that a sodium chloride extract-soluble, but water-insoluble, lipase preparation (as distinct from esterase preparation) was obtained from castor beans.[10] An analogous lipase preparation, but soluble in water, was obtained from soy beans.[11] Studies of the solubility changes of these preparations on simple treatments did not lead to any conclusions because of the relative instability of the preparations.

Whether the change of a soluble enzyme preparation into an insoluble one by simple treatment is considered to be adsorption, or chemical interaction according to definite stoichiometrical ratios, may, at the present time, be looked upon as an academic

[10] Falk, K. G., *J. Am. Chem. Soc.*, 1913, xxxv, 1904.
[11] Falk, K. G., *J. Am. Chem. Soc.*, 1915, xxxvii, 649.

question. The writers prefer to speak of it as chemical interaction, but the exact significance to be attached to the words, and this applies as well to those who prefer to speak of the changes as adsorption, must wait for a more complete and satisfactory knowledge of the chemical structures of the substances involved, and the changes accompanying the phenomena.

The writers wish to express their thanks to Mr. Isaac Lorberblatt for his aid in preparing the banana extracts used in this work.

CONCLUSIONS.

The properties of the gels obtained from banana extract by treatment with calcium salts and alkali or with pancreatin and of banana sucrase were studied. It was shown that different substances were involved in the gel formations and in the enzyme actions, although boiling the solutions destroyed both properties.

The formation of the gel and the conditions for dissolving it without inactivation of the enzyme were described.

The relation of the results obtained to those obtained by others, as well as their bearing upon some general enzyme questions, is discussed.

ESTERIFICATION OF CREATINE.*

By ARTHUR W. DOX and LESTER YODER.

(From the Department of Chemical Research, Parke, Davis and Company, Detroit.)

Many of the amino-acids resulting from hydrolysis of proteins have been esterified by treatment with absolute alcohol and dry hydrogen chloride. The amino-acid, usually amphoteric in reaction because of the simultaneous presence of an amino and a carboxyl group, becomes strongly basic when the carboxyl is esterified, and the amino group readily adds an equivalent of mineral acid to form a stable crystalline salt. Fractional distillation *in vacuo* of these basic esters constitutes Fischer's well known method of separating certain of the amino-acids present in the acid hydrolysate of proteins.

From glycine, or aminoacetic acid, the simplest of the amino-acids, the hydrochloride of the ethyl ester is readily obtained in large crystals. Creatine, or methylguanidine-acetic acid, may be regarded as a substituted glycine. The conversion of creatine into creatinine by loss of water is readily effected by treatment with mineral acid. It was significant, therefore, to find that treatment of creatine with absolute alcohol and dry hydrogen chloride, a condition ideal for the removal of water, resulted not in the ring closure but rather in esterification of the carboxyl group. Esters were readily obtained in this way with methyl, ethyl, and *n*-butyl alcohols. Since no esters of creatine are described in the literature, the following derivatives may be of interest.

Creatine Methyl Ester Hydrochloride.—10 gm. of creatine, previously dried at 100°, were suspended in 100 cc. of absolute methyl alcohol and dry hydrogen chloride was passed in. In a short time

* Published as Contribution No. 17 from the Department of Chemical Research, Parke, Davis and Company.

the creatine had completely dissolved. The addition of hydrogen chloride was continued until the mixture was saturated at room temperature. On standing over night no crystals separated. 2 volumes of ether were added and the mixture was cooled in ice water. Within half an hour an abundance of slender needle-shaped crystals had separated. These were filtered and washed with ether, then recrystallized from ethyl alcohol. The yield was 9.0 gm. of slender needles, very soluble in water, moderately soluble in alcohol, and insoluble in ether. The substance has a strongly saline taste. It melted at 139-140° with evolution of gas, and then solidified. When gradually heated in an open flask in an oil bath the substance sinters together without melting. In this reaction methyl alcohol is given off and ring closure occurs, resulting in the formation of creatinine hydrochloride. This was identified by the nitrogen content and by the Jaffé reaction. A dilute solution of the ester hydrochloride gives with picric acid a copious separation of fine yellow needles. The ester hydrochloride gave the following figures:

Analysis.
$C_5H_{11}N_3O_2 \cdot HCl$.   Calculated.   N 23.44,   Cl 19.56.
                        Found.   " 23.31, 23.38; " 19.69, 19.52.

Creatine Ethyl Ester Hydrochloride.—10 gm. of dry creatine were suspended in 100 cc. of absolute ethyl alcohol and dry hydrogen chloride was passed in. The creatine dissolved as in the preceding preparation, but as the mixture became saturated with hydrogen chloride, the ester hydrochloride crystallized out without the addition of ether. After recrystallizing from alcohol 11.5 gm. of needle-shaped crystals were obtained. The substance melted at 163° with evolution of gas, leaving a white solid residue which gave the Jaffé test for creatinine. It is readily soluble in water, moderately soluble in alcohol, and insoluble in ether, and forms a difficultly soluble crystalline picrate.

Analysis.
$C_6H_{13}N_3O_2 \cdot HCl$.   Calculated.   N 21.48,   Cl 18.16.
                        Found.   " 21.49, 21.56; " 17.68, 17.69.

Creatine n-Butyl Ester Hydrochloride.—This was prepared in the same manner as the above, using n-butyl in place of ethyl alcohol. The product crystallized from the mixture in flat needles.

Recrystallized from ethyl alcohol, they melted at 138° and decomposed at about 150°, leaving a solid residue of creatinine hydrochloride. The yield was 11.0 gm. Like the two preceding derivatives, this ester salt is very soluble in water, moderately soluble in alcohol, insoluble in ether, and forms a difficultly soluble picrate.

Analysis.

$C_5H_{17}N_3O_2 \cdot HCl$.   Calculated.   N 18.79,   Cl 15.98.

                Found.   " 19.39, 19.32; " 15.95, 16.30.

Isopropyl alcohol failed to react with creatine under the conditions of the above experiments. This is not surprising in view of the greater rate of esterification of primary as compared with secondary alcohols.

A COLORIMETER FOR BICOLORIMETRIC WORK.*

By VICTOR C. MYERS.

*(From the Department of Biochemistry, New York Post-Graduate Medical
School and Hospital, New York.)*

(Received for publication, October 11, 1922.)

There are many purposes for which a colorimeter employing a
single color standard is inadequate. One of the best illustrations
is the colorimetric determination of the hydrogen ion concentra-
tion. As ordinarily carried out this determination is cumber-
some and rather crude, since it has been necessary to employ a
series of tubes with graduated pH valves as standards, owing to
the fact that here one is dealing with combinations of two colors
instead of a single color.

It has been pointed out[1] that with the use of two wedges in a
modified Hellige colorimeter, it is possible to match all the shades
of color in a given indicator from the acid to the alkaline side,
when one wedge is filled with an acid solution of the dye and the
other with an alkaline solution, thus reducing the number of
standards employed with a single indicator to two. These may
be made with buffer solutions of a definite pH, or of solutions
of the indicator made sufficiently acid or alkaline to produce a
complete change in the color of the indicator. Where it is de-
sired to read very small differences in pH over a limited range the
two standards can best be made of a definite pH just outside of
the range to be covered.

Barnett and Barnett[2] and Gillespie[3] have employed similar
principles in the colorimetric measurement of the hydrogen ion

---

[1] Myers, V. C., *Proc. Soc. Exp. Biol. and Med.*, 1921–22, xix, 78.

[2] Barnett, G. D., and Barnett, C. W., *Proc. Soc. Exp. Biol. and Med.*,
1920–21, xviii, 127.

[3] Gillespie, L. J., *J. Bact.*, 1921, vi, 399.

concentration. The former authors employ a low, narrow, rectangular glass box having a diagonal glass partition, one side being used for the acid and the other for the alkaline solution of the indicator, while the latter achieves the same result by having a small movable cup fitted over the plunger but inside the cup of a Duboseq type colorimeter.

It is believed that the use of wedges which are individually movable provides a much more flexible system. The reading of the wedge containing the dominant color of the dye, *e.g.* the red in phenol red, characterizes the hydrogen ion concentration, the yellow wedge being employed simply to obtain a correct color match. This being the case it may also be employed to correct for any slight error due to extraneous yellow pigment in the unknown.

Modified Hellige Colorimeter.

In our first attempt to utilize the above principle the Autenrieth-Königsberger colorimeter of Hellige was modified so as to provide for a second wedge[4] and a magnifying eyepiece. This instrument is illustrated in Fig. 1. It will be noted that space has been so economized as to take care of the two wedges in the box of a standard Hellige colorimeter. The two adjustment screws are placed on either side of the instrument to permit the use of both hands in matching the colors. With pure solutions this instrument works excellently. For the highest degree of accuracy, however, it seemed desirable that a metal construction should be employed.

New Wedge Colorimeter.

A new wedge colorimeter,[5] having a number of advantages over the instrument illustrated in Fig. 1, is shown in Figs. 2 and 3.

[4] Sometime after our instruments had been constructed our attention was called to a colorimeter described by Hellige and Co. which contained several wedges. The design of this instrument is similar to that of their single wedge colorimeter, except that the box is much deeper to allow for the extra wedges. The uses which were suggested for this instrument are quite different, however, from those mentioned in the present paper, the extra wedges being employed chiefly to absorb interfering colors.

[5] These instruments were constructed by E. Leitz, Inc., 60 East 10th Street, New York City, who are arranging to manufacture them. The author wishes to express his appreciation of their helpful cooperation in the development of the instruments.

Briefly this instrument comprises a brass box with heavy metal base. It is 30 cm. in height, and contains a rack and pinion arrangement for three wedges, the movement of the wedges being entirely within the closed box. Readings are taken from 100 mm. (adjustable) scales which emerge from the top of the instrument

Fig. 1.

as the wedges are raised. The instrument is provided with an eyepiece containing magnifying lens. The Helmholtz prisms used in the Hellige instrument have been replaced with the type of prisms employed in instruments made on the Duboseq pattern. With these prisms the light passes more nearly through the center

of the wedges and cup, furnishing a better field for comparison. A milk-glass plate in back allows for the entrance of light. If desired, a small lamp box may be substituted for this. The lamp box as now constructed uses only reflected light, which passes

Fig. 2.

through a thick daylite glass. The small nitrogen bulb is set below the field of vision, the reflecting surface being covered with aluminum paint. The light obtained from the present lamp compares favorably with daylight in quality, although somewhat greater in intensity. A door at the side of the instrument gives

access to the wedges and to the cup for the unknown which is
mounted on it (see Fig. 3). Two wedges provide for bicolorimetric
work as in the pH determination. However, to obtain a perfect
match with unknown solutions which are slightly turbid or

Fig. 3.

colored a third wedge may be used. To secure an even field it
is sometimes necessary to counterbalance the greater thickness of
glass on the wedge side of the field. Provision has been made
for this by attaching to the rack carrying the cup for the unknown
a clip, which will hold one or more glass plates.

Wedges made according to the Hellige design are constructed with such an angle that, when a single wedge is used, the 100 mm. scale should read the strength of the unknown in per cent; *i.e.*, with a reading of 37 mm. the unknown should be 37 per cent the strength of the standard. Practically it is very difficult to manufacture wedges which are exactly correct, and it is best simply to calibrate each wedge. However, both the wedges and cup are provided with adjustments, the cup adjustment having a vernier scale. When the angle of the wedge is such as to require only slight correction, these two adjustments will permit sufficient correction to secure accurate percentage readings.

Accuracy of Reading with the Instrument.

The chart shown in Fig. 4 is presented to illustrate the reading accuracy of the instrument. Standards were prepared to cover the same range as that employed by Cullen[6] in his colorimetric pH determination in blood. The standard solutions for the two wedges were made with pH values of about 8.0 and 6.8. For the alkaline wedge 1.1 cc. of M/15 primary and 18.9 cc. of M/15 secondary phosphate (Merck's special reagent) were employed, while for the acid wedge 10.2 cc. of primary and 9.8 cc. of secondary phosphate were used. 2 cc. of 0.02 per cent phenol red were added to both solutions. For the calibration of the wedges, nine different standards were employed covering the pH range from 7.0 to 7.8, the standards differing by 0.1 pH. 5 cc. portions were prepared in Pyrex test-tubes from the M/15 phosphates, and 0.5 cc. of the phenol red solution was added to each. It will be noted that none of the readings fall more than one place away from the curve plotted, the value of one place on the scale being 0.012 pH. It is believed that under favorable conditions the reading accuracy of the instrument for the above range of pH should be well within ± 0.02.

Uses of the Instrument.

It is believed that this instrument will be found well suited to the colorimetric determination of the hydrogen ion concentration in various biological fluids such as urine, gastric contents, blood,

---

[6] Cullen, G. E., *J. Biol. Chem.*, 1922, lii, 501.

bacteriological culture media, etc. The instrument also serves excellently for the Marriott alveolar carbon dioxide test. It is a matter of common observation that in the phenolsulfonephthalein renal function test it is rarely possible to obtain an exact color match, probably because certain salts present in the urine prevent

Fig. 4. Readings made with wedge containing dominant color (alkaline, pH 8.0, phenol red solution).

a complete change in the color of the indicator on the addition of alkali. By using an acid (yellow) 'phthalein standard in conjunction with the regular alkaline 'phthalein standard it is always possible to obtain an exact color match. If desired, correction may be made for the rather small error introduced by

the "off" color. For the Marriott alveolar carbon dioxide test
and the Rowntree-Geraghty 'phthalein test, the instrument
illustrated in Fig. 1 is adequate, but for the more exact bicolori-
metric hydrogen ion concentration work the new instrument
(Figs. 2 and 3) is to be preferred.

SUMMARY.

A new colorimeter which has been designed chiefly for such
bicolorimetric work as the colorimetric pH determination is
described. Standards are carried in wedges, one, two, or three
of which may be employed at the same time. With one wedge
the instrument may be used as an ordinary colorimeter. The
second wedge provides for bicolorimetric work. To obtain a
perfect match with unknown solutions which are slightly turbid
or colored a third wedge may be used.

THE RELATIONS EXISTING BETWEEN ARTERIAL AND VENOUS BLOOD OF THE DOG WITH SPECIAL REFERENCE TO THE PLASMA CHLORIDES.

By EDWARD A. DOISY and J. W. BECKMANN.

(*From the Laboratories of Biological Chemistry, Washington University School of Medicine, St. Louis.*)

In this paper we are presenting a study of the relations existing between the oxygen and carbon dioxide of blood and between the carbon dioxide and chlorides of the plasma of blood drawn simultaneously from the femoral artery and vein of the dog. Our primary purpose in undertaking this study was to determine if the chloride shift occurs *in vivo* as well as *in vitro*. Douglas and Haldane (1922) have obtained data upon the living subject which they interpret as evidence of the effect of oxygen unsaturation upon the capacity of the blood to bind carbon dioxide. But this work dealt with the blood as a unit, whereas our work on the chloride shift deals with it in sections. It is obvious that when blood takes up carbon dioxide if there were no mechanism whereby the efficiency of the poorly buffered plasma could be increased, there would be the opportunity for large variations in the hydrogen ion concentration of the plasma. We have previously published results of *in vitro* work on the "loaned buffer" (Doisy, Eaton, and Chouke, 1922), and our present studies indicate that the hemoglobin loans buffer to the plasma by means of the shift of hydrochloric acid *in the body*, as well as in the test-tube.

Although our chief purpose was to study the shift of chlorides, it seemed desirable to secure additional information on the arterial to venous change of blood. Few papers have dealt with this topic. Both Stadie (1919) and Harrop (1919) have studied arterial and venous blood of men, and recently Peters, Barr, and Rule (1920-21) have published more extensive studies. In the last two papers the relation of carbon dioxide taken up to oxygen lost has not

consistently yielded normal respiratory quotients. It appeared probable to us that this was occasioned chiefly by technical and physiological difficulties in obtaining the blood samples. We have, therefore, eliminated some of these difficulties by carrying out our experiments on dogs in which the blood vessels were exposed before attempting to draw samples.

Still another point considered was the distribution of carbon dioxide taken up in the venous blood between the corpuscles and plasma. Means and his collaborators (1920–21) found that only about 10 per cent of the added CO_2 was carried in the plasma. Some of our earlier *in vitro* work did not agree with this in that it indicated that added carbon dioxide was almost equally distributed between cells and plasma. Certainly, this arrangement would fit in better with the idea of a uniform C_H of corpuscles and plasma or with an approximately constant slight difference of C_H (Campbell and Poulton, 1920–21). With equal volumes of corpuscles and plasma, it is necessary for the bicarbonate to be increased in both phases at nearly the same rate if the relation between the hydrogen ion concentration of the cells and plasma is to be maintained. More specifically the increase must bear a relationship to the solubility coefficients of CO_2 in corpuscles (0.45) and in plasma (0.54) if the ratio $\dfrac{H_2CO_3}{NaHCO_3}$ is to remain constant. Our data indicate an approximately equal distribution of the CO_2 taken up when the blood becomes venous.

This part of our work required determinations of the volume occupied by the corpuscles of the blood. These data may be utilized to test the idea that cells swell when treated with an increased tension of carbon dioxide. Von Limbeck (1894-95) noted this phenomenon and others (Mukai, 1921; Moore, 1916) have studied it on isolated tissues. In a previous paper, Doisy and Eaton (1921) have related it to the greater buffer value of the cells and the indiffusibility of the cell buffers. While the values obtained are not strikingly constant, which may be due to errors of technique and uncontrolled physiological factors, the data indicate a transfer of water from plasma to cells when the blood becomes venous.

EXPERIMENTAL PROCEDURE.

In order to avoid the effects of ether (Van Slyke, Austin, and Cullen, 1922), local anesthesia with cocaine or butyn was used. In a few of our experiments in which very nervous dogs were used, morphine was given subcutaneously about an hour before putting the animal on the table. The femoral artery and vein were exposed under the local anesthetic and needles which were connected with 25 cc. pipettes inserted into the vessels. The pipettes contained oil and the same weight of potassium oxalate. It seemed necessary to secure exactly the same concentration of oxalate in both the venous and arterial blood to avoid any shift of water between cells and plasma. In view of the nature of our experiments, we attempted to draw the arterial and venous blood simultaneously during a period of even, regular respiratory movements, and in most of our experiments this was successful.

Blood was transferred under oil to hematocrit and centrifuge tubes and centrifuged in stoppered tubes at high speed to obtain plasma for analysis and the volume of the corpuscles. In the meantime, the blood was analyzed for carbon dioxide and oxygen. After completion of the centrifugation the plasma was analyzed for carbon dioxide and chlorides.

Although this work was begun during the summer of 1921, the paper was not submitted for publication until the experiments were repeated using the improved methods of Van Slyke and Stadie (1921) for the analysis of carbon dioxide and oxygen. Chlorides were determined by a modification of the Austin-Van Slyke (1920) procedure. Using very carefully controlled conditions our duplicate determinations rarely differed by as much as 0.0004 M and generally the difference was no greater than 0.0002 M.

DATA AND DISCUSSION.

In Table I we have collected our data on the chloride and carbon dioxide concentrations of arterial and venous plasma. The average difference of carbon dioxide is 0.00196 M, while that of the chloride is 0.00138 M. The difference in these two values may be explained in several ways. (1) The carbon dioxide values are expressed as total CO_2 and not bicarbonate. In a recent paper (1922) we have shown that in man about 10 per cent of the car-

bon dioxide carried by blood in becoming venous is due to physically dissolved CO_2 due to the greater tension existing in the capillaries than in the arterial blood. This uncombined CO_2 does not utilize base and to make the results comparable should

TABLE I.

Comparison of the Carbon Dioxide and Chloride Concentration of the Plasma of Arterial and Venous Blood.

Experiment No.	Arterial total CO_2.	Venous total CO_2.	Difference, Column 3 − Column 2.	Arterial NaCl.	Venous NaCl.	Difference, Column 5 − Column 6.
	M	M	M	M	M	M
1*	0.0209	0.0233	0.0024	0.1087	0.1075	0.0012
2	0.0230	0.0242	0.0012	0.1125	0.1120	0.0005
3	0.0235	0.0244	0.0009	0.1155	0.1148	0.0007
4*	0.0221	0.0240	0.0019	0.1170	0.1154	0.0016
5*†	0.0254	0.0254		0.1136	0.1136	
6	0.0247	0.0256	0.0009	0.1064	0.1055	0.0009
7	0.0193	0.0219	0.0026	0.1153	0.1137	0.0016
8	0.0205	0.0223	0.0018	0.1117	0.1102	0.0015
9†	0.0225	0.0239	0.0014	0.1141	0.1132	0.0009
10†	0.0228	0.0228		0.1102	0.1103	
11	0.0220	0.0232	0.0012	0.1124	0.1112	0.0012
12*	0.0218	0.0240	0.0022	0.1148	0.1132	0.0016
13*	0.0218	0.0222	0.0004	0.1142	0.1132	0.0010
14	0.0181	0.0200	0.0019	0.1132	0.1117	0.0015
15*	0.0185	0.0203	0.0018	0.1159	0.1150	0.0009
16	0.0232	0.0242	0.0010	0.1102	0.1092	0.0010
17*	0.0243	0.0269	0.0026	0.1133	0.1113	0.0020
18*	0.0217	0.0267	0.0050	0.1111	0.1081	0.0030
19*	0.0273	0.0310	0.0037	0.1086	0.1064	0.0022
20*	0.0252	0.0278	0.0026	0.1105	0.1087	0.0018
21	0.0275	0.0294	0.0019	0.1057	0.1042	0.0015
22	0.0221	0.0234	0.0013	0.1094	0.1088	0.0006
Mean........			0.00196			0.00138

* Morphine used to quiet dog.
† Omitted in obtaining mean results.

be subtracted from our mean difference of plasma carbon dioxide. (2) The "self-possessed" buffer value of plasma while small (3 to 5 per cent) in the arterial to venous change should likewise be subtracted from 0.00196. Neither of these subtractions is possible because the pH of the arterial and venous blood of our

dogs was not determined. (3) No account is taken of any concentration effect, due to an increase of size of the corpuscles. We have avoided making this correction because of the uncertainties involved, chief of which is the effect of the cells of the muscle tissue in the equilibrium. In another series of experiments we have found that stimulation of the sciatic nerve between the taking of the arterial and venous blood causes a huge loss of water from the plasma of the latter. This, however, does not detract from the main point, namely the demonstration that the chloride shift occurs *in vivo*.

The relation between the plasma chlorides of arterial and venous blood is a bit more complicated than in the *in vitro* experiments in which the hemoglobin is kept at the same degree of oxygen saturation. In the former case there is an increased capacity of the blood to bind carbon dioxide without change of pH (Henderson, 1921) which may be expressed by saying that for any pH the numerical value of the ratio $\dfrac{BHbO_2}{HHbO_2}$ is greater than that of the ratio $\dfrac{BHb}{HHb}$. This increment of carbon dioxide, the base for which is provided by a reduction of oxyhemoglobin, is distributed between cells and plasma. In addition there is a certain quota due to the change of pH. Both of these reactions produce a migration of hydrochloric acid into the cells with a consequent increase of the bicarbonate of the plasma. This lump sum plus the physically dissolved carbon dioxide increase is what we have measured in our experiments.

In all our experiments with the exception of Nos. 5 and 10 we have found a migration of hydrochloric acid. The results of these two experiments can probably be explained by our failure to obtain the blood simultaneously and an alteration of the respiration between the arterial and venous puncture. We have omitted No. 9 from our consideration, even though it apparently shows a chloride migration, for the same reasons. Our conclusion, then, is that the migration of hydrochloric acid which had previously been studied in the test-tube actually occurs in the body under normal physiological conditions.

Our results on blood respiratory quotients which are given in Table II show that out of seventeen experiments eight values fall between 0.70 and 1.00. Of the remaining nine only three lie outside of the limits 0.67 to 1.05, values which have frequently been observed in studying the gaseous exchange of man. Two of the other three results are low, while the third is very low, and may be explained by the failure of our technique which has already been

TABLE II.

The Loss of Oxygen and the Increase of Carbon Dioxide when Blood Changes from Arterial to Venous Condition.

Experiment No.	Arterial total CO_2.	Venous total CO_2.	Difference, Column 3 − Column 2.	Arterial total O_2.	Venous total O_2.	Difference, Column 5 − Column 6.	Blood respiratory quotient, Column 4 / Column 7
	vol. per cent	vol. per cent	vol. per cent	vol. per cent	vol. per cent	vol. per cent	
1	39.2	43.2	4.0	20.3	14.4	5.9	0.67
2	41.4	43.6	2.2	21.1	19.0	2.1	1.05
3	43.9	46.8	2.9	16.5	12.8	3.7	0.78
4	40.1	44.8	4.7	23.7	19.2	4.5	1.05
5	47.8	48.1	0.3	15.0	11.8	3.2	0.10
6	43.9	46.7	2.8	23.5	19.7	3.8	0.74
7	33.8	39.2	5.4	27.2	19.3	7.9	0.68
11	40.8	44.9	4.1	18.0	12.1	5.9	0.70
12	39.7	44.4	4.7	22.1	15.1	7.0	0.67
14	32.4	35.5	3.1	22.5	17.9	4.6	0.67
15	33.4	37.1	3.7	22.3	17.8	4.5	0.82
16	40.0	42.6	2.6	25.5	22.8	2.7	0.97
17	44.1	53.2	9.1	22.1	10.1	12.0	0.76
18	39.6	50.6	11.0	20.6	2.5	18.1	0.61
20	44.9	50.1	5.2	21.6	14.3	7.3	0.71
21	47.9	51.6	3.7	22.3	15.9	6.4	0.58
22	38.4	41.2	2.8	18.6	15.6	3.0	0.93

referred to. This result has been included in our table merely to indicate the degree that extraneous influences may affect the relations of arterial and venous blood.

Table III indicates the distribution of carbon dioxide between cells and plasma. Of the amount taken up in the passage through the capillaries 52 per cent was found in the cells. The variations (37 to 75 per cent) of the individual experiments are not greater than should be expected when one considers the various deter-

minations involved in which the difference involved in any set is rarely more than 3 or 4 volumes per cent. In Experiment 18 in which the difference between the arterial and venous blood was very large 46 per cent of the total CO_2 taken up was held in the cells. This value agrees fairly well with some of our *in vitro* experiments (unpublished data).

TABLE III.

The Amount of Carbon Dioxide Carried in the Corpuscles and Plasma of Venous Blood. *

Experiment No.	Arterial plasma.	Venous plasma.	Difference, Column 3 − Column 2.	Arterial corpuscles.	Venous corpuscles.	Difference, Column 6 − Column 5.	CO_2 carried by corpuscles. Per cent of total.
	cc. CO_2	cc. CO_2	cc. CO_2	cc. CO_2	cc. CO_2	cc. CO_2	
1	23.7	26.2	2.5	15.5	17.0	1.5	37.5
2	27.7	28.8	1.1	13.7	14.8	1.1	50.0
3	32.6	33.7	1.1	11.3	13.1	1.8	62.1
4	28.3	30.7	2.4	11.8	14.1	2.3	48.9
6	27.5	28.9	1.4	16.4	17.8	1.4	50.0
7	18.1	21.3	3.2	15.7	17.9	2.2	40.8
11	28.1	30.2	2.1	12.7	14.7	2.0	48.8
12	24.3	26.9	2.6	15.4	17.5	2.1	44.7
14	19.4	20.9	1.5	13.0	14.6	1.6	51.6
15	20.2	21.1	0.9	13.2	16.0	2.8	75.7
16	23.6	24.5	0.9	16.4	18.1	1.7	65.4
17	29.5	32.3	2.8	15.0	17.1	2.1	42.9
18	25.3	31.2	5.9	14.3	19.4	5.1	46.2
20	25.2	27.4	2.2	19.7	22.7	3.0	57.9
21	30.1	31.9	1.8	17.8	19.7	1.9	51.4
22	27.7	28.7	1.0	10.7	12.5	1.8	64.3
Mean							52.4

*In 100 cc. of blood.

Our mean value of 52 per cent is very much smaller than that given by Smith, Means, and Woodwell (1920–21). It might be argued that this is due to their use of men and our use of dogs, but we do not believe this to be true. Our *in vitro* experiments on human blood indicate that about 45 per cent of the carbon dioxide added by increases of carbon dioxide tension is taken up by the cells. However, we do agree with their conclusion that the cells are mainly responsible for the transport of carbon dioxide (Doisy, Briggs, Eaton, and Chambers, 1922).

Our data dealing with the increase in volume occupied by the corpuscles when the blood becomes venous are given in Table IV. These data are not entirely concordant, but in general show that the corpuscles of the venous blood occupy a larger volume than those of the arterial blood. Some physiological factors which may vitiate the accuracy of these determinations have already been mentioned.

TABLE IV.

The Volume Occupied by the Corpuscles in the Arterial and Venous States.

Experiment No.	Arterial blood, per cent plasma.	Venous blood, per cent plasma.	Decrease, per cent venous plasma.
1	50.5	50.1	0.4
2	53.7	53.1	0.6
3	61.9	61.8	0.1
5	61.8	61.1	0.7
6	49.8	50.3	−0.5
7	41.7	43.3	−1.6
9	49.8	48.7	1.1
10	55.1	55.5	−0.4
11	57.1	58.2	−1.1
12	50.1	50.0	0.1
14	47.9	46.9	1.0
15	48.7	46.5	2.2
16	45.5	45.1	0.4
17	54.3	53.5	0.8
18	52.1	52.0	0.1
19	59.9	58.2	1.7
20	44.7	44.1	0.6
21	48.9	48.5	0.4
22	55.7	54.7	1.0
Mean...			+0.35

We consider that the explanation of this phenomenon must be sought in part, at least, in the alterations of osmotic pressure. It seems probable to us that as base is taken from the buffers to bind carbon dioxide more ions are produced in the solution. This increase occurs more rapidly in the cells due to their greater buffer value, and consequently water is withdrawn from the plasma to maintain the already existing relations. This statement should not be construed to mean that we consider the osmotic pressure of the cells to equal that of the plasma, for there is some evidence to the contrary.

SUMMARY.

It appears that a perfectly executed experiment on arterial and venous blood should show the following points: (1) Migration of chlorides into the cells; (2) normal blood respiratory quotient; (3) approximately equal distribution of the added CO_2 between cells and plasma; and (4) larger volume occupied by the corpuscles of venous blood.

Consideration of our seventeen complete experiments in the tables shows that Experiments 3, 15, 17, 20, and 22 fulfill these conditions. It may be noted that in our later work which had the benefit of the practice of the earlier experiments the number of successful experiments is much larger. If it is permissible to extend the limits of normal respiratory quotients to from 0.67 to 1.05, then we have four more experiments which may be classed as satisfactory. A positive result in twenty out of twenty-two experiments supports the occurrence of a migration of hydrochloric acid in the blood in the body.

BIBLIOGRAPHY.

Austin, J. H., and Van Slyke, D. D., *J. Biol. Chem.*, 1920, xli, 345.

Campbell, J. M. H., and Poulton, E. P., *J. Physiol.*, 1920-21, liv, 152.

Doisy, E. A., and Eaton, E. P., *J. Biol. Chem.*, 1921, xlvii, 377.

Doisy, E. A., Briggs, A. P., Eaton, E. P., and Chambers, W. H., *J. Biol. Chem.*, 1922, liv, 305.

Doisy, E. A., Eaton, E. P., and Chouke, K. S., *J. Biol. Chem.*, 1922, liii, 61.

Douglas, C. G., and Haldane, J. S., *J. Physiol.*, 1922, lvi, 69.

Harrop, G. A., *J. Exp. Med.*, 1919, xxx, 241.

Henderson, L. J., *J. Biol. Chem.*, 1921, xlvi, 411.

von Limbeck, R., *Arch. exp. Path. u. Pharmacol.*, 1894-95, xxxv, 309.

Moore, A. R., *Am. J. Physiol.*, 1916, xli, 137.

Mukai, G., *J. Physiol.*, 1921, lv, 356.

Peters, J. P., Barr, D. P., and Rule, F. D., *J. Biol. Chem.*, 1920-21, xlv, 489.

Smith, L. W., Means, J. H., and Woodwell, M. N., *J. Biol. Chem.*, 1920-21, xlv, 245.

Stadie, W. C., *J. Exp. Med.*, 1919, xxx, 215.

Van Slyke, D. D., Austin, J. H., and Cullen, G. E., *J. Biol. Chem.*, 1922, liii, 277.

Van Slyke, D. D., and Stadie, W. C., *J. Biol. Chem.*, 1921, xlix, 1.

THE NON-PROTEIN ORGANIC CONSTITUENTS IN THE BLOOD OF MARINE FISH.*

By W. DENIS.

(From the Laboratory of Physiological Chemistry of the School of Medicine, Tulane University, New Orleans.)

Nine years ago I published (1) analyses of the blood of several species of fish occurring on the north Atlantic coast. Since this time great advances have been made in the accuracy of the technique of blood analysis; a field still young, but which in 1912, the time at which my first work was attempted, was in its infancy. With the numerous improvements in technique which have come with the passing years our ideas as to what constitutes "normal" values for the non-protein constituents of human blood have undergone radical change, and, such being the case it has seemed worth while to repeat some of my former observations on the blood of marine fish, as it would appear probable that at least a portion of the figures obtained by the relatively crude methods available at that time are in need of revision.

The analytical methods used were as follows: Non-protein nitrogen, urea, creatinine, and glucose by the Folin-Wu (2) procedures; amino nitrogen by Folin's method (3); and uric acid by the direct method of Benedict (4).

All animals used were bled as soon as brought in from the traps; blood was secured by cutting off the tail and collecting from the caudal vessels, without the use of an anticoagulant. The results obtained on the blood of seven species of fish are presented in Table I.

In comparing the analytical figures collected in Table I with my own earlier work on the blood of marine fish and the values published by Wilson and Adolph (5) on fresh water fish, which investigations constitute the only chemical studies of fish blood in

---

* This work was carried on during the Summer of 1922 in the Marine Biological Laboratory at Woods Hole.

which modern methods have been employed, it will be noted that the figures for non-protein nitrogen and urea are along the same general level as are the results previously published. The non-protein nitrogen in the blood of elasmobranch fish in both series of results lies in the neighborhood of 1,000 mg. per 100 cc. of blood, and the urea nitrogen at about 800 mg.

In the case of the teleost fish my former observations showed results varying from 40 to 90 mg. of non-protein nitrogen per 100 cc. of blood (average about 65 mg.), whereas in the present series

TABLE I.

Non-Protein Constituents in the Blood of Fish.

	Per 100 cc. blood.							
	Total non-protein N.	Urea N.	Amino N.	Creatinine.	Creatine.	Uric acid.	N H₂-N	Blood sugar.
	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.
Dogfish (*Mustelis canis*)	1,000	805	28	6.6	25	1.0	1.6	80
Dusky shark (*Carcharinus obscurus*)	1,000	866	31	6.0	27	1.3	1.7	97
Sand shark (*Carcharias littoralis*)	1,010	800	39	7.6	36	1.1		
Sculpin (*Myoxocephalus aenens*)	56	9	28	1.1	6.0	4.0		
Tantog (*Tantoga anitis*)	60	7.8	26.6	1.1	6.2	4.4		
Cunner (*Tantogolabrus*)	63	10	28	1.3	6.6	4.2		
Menhaden (*Brevoortia tyrannus*)	73	8.0	21	1.5	6.0	4.3	1.3	90

the results vary from 56 to 73 mg. The values obtained for urea nitrogen in teleost blood varied in my earlier observations from 8 to 20 mg., and in the present series from 7.8 to 10.

The values reported by Wilson and Adolph for the fresh water teleost are distinctly lower than the above; *i.e.*, non-protein nitrogen 29 to 59, and urea nitrogen from 1.1 to 16.0 mg.

Striking differences are found in the creatinine and creatine content of the elasmobranch and teleost bloods, the high creatinine and creatine content of the former (creatinine 6.0 to 7.6, and creatine 25 to 36 mg.) being in marked contrast to the values obtained for the latter type (creatinine 1.1 and 1.3, and creatine 6.0 to 6.6 mg.), values which closely resemble those found in human blood.

A practical reversal of this condition is to be noted in the occurrence of uric acid in the blood of the two classes, in the elasmobranches about 1 mg. and in the teleosts about 4 mg. In my first publication the statement was made that elasmobranch blood is free from uric acid while the quantity found in teleost blood amounted to only 1.0 to 1.5 mg., results which were doubtless due to the crude analytical technique available at that time.

The level of amino nitrogen was found to be about the same in the blood of both elasmobranches and teleosts varying from 21.0 to 31 mg. In my earlier work no determinations of amino nitrogen were made, but in the blood of fresh water fish Wilson and Adolph have reported figures varying from 17 to 34 mg.

The figures of ammonia and blood sugar require special comment. In my earlier paper on fish blood I reported a content of ammonia nitrogen of from 1.0 to 5.5 mg. per 100 cc. of blood; Wilson and Adolph found 0.9 to 1.8 mg. in fresh water fish. These results were obtained by the aeration method. Of late years additional work on the ammonia method has brought to light many formerly unsuspected sources of error (6). In view of the large amount of ammonia apparently present in fish blood I have used a method of procedure which I tried out some years ago on human blood but which was abandoned because it was found inadequate to cope with the extremely small amount of ammonia present in this fluid. The general principle consists of the precipitation of the proteins of blood with m-phosphoric acid (7) and the extraction of the ammonia from the filtrate so obtained by means of permutit.

The details of my procedure are as follows: The blood was collected without the addition of any anticoagulant; to this were immediately added $1\frac{1}{2}$ volumes of distilled water and $\frac{1}{2}$ volume of a 20 per cent freshly prepared solution of m-phosphoric acid. A rubber stopper was then inserted and the flask shaken vigorously and continuously for 10 minutes to induce rapid precipitation. The mixture was then poured on a dry filter and an aliquot of this filtrate (usually 15 cc.) was shaken for 5 minutes with 2 gm. of permutit, which just before use had been freed from traces of ammonia by treatment successively with alkali, acid, and distilled water (8). The supernatant liquid was then poured off, the permutit washed twice with distilled water, thus treated with 10 cc. of 0.5 per cent sodium hydroxide after standing for 5 minutes with

more distilled water and 3 cc. of Nessler's solution, was finally made up to a volume of 50 cc., and compared in the colorimeter with a suitable standard. The standard solution found most convenient consisted of ammonium sulfate solution equivalent to 0.1 mg. of nitrogen, 20 cc. of 0.5 per cent sodium hydroxide, and 6 cc. of Nessler's solution, and the whole was finally made up with distilled water to a volume of 100 cc. I have confined the use of the above method to observations on the blood of three species, shark, dogfish, and menhaden, as these were the only varieties of which the supply was sufficiently abundant to furnish material for a reasonably large number of determinations.

As will be seen from Table I the values obtained lie between 1.7 and 1.3 mg., figures much smaller than those obtained in my earlier observations, and approaching those found by Wilson and Adolph for fresh water fish, but still greatly in advance of the ammonia values reported for human blood.

A moderate number of observations have been made regarding the reducing bodies in fish blood, ordinarily classed under the term "blood sugar." As discussions concerning the earlier work on this subject are to be found in the recent papers of Lang and Macleod (9) and of Scott (10) further comment is superfluous.

As a considerable diversity of opinion exists among investigators regarding the influence of asphyxia on the blood sugar in fish the following experiment was tried with the dogfish. The animals were obtained as soon as they were brought in from the traps and were placed in the laboratory in a large tank of running sea water. After being allowed to remain over night in this tank, during which time they had apparently recovered from the handling incident to capture, the fish were removed quickly, the tail was severed as promptly as possible and a small amount of blood (2 cc.) was collected. The blood which continued to drip from the severed vessels was received in 2 cc. fractions, the time of collection being noted, and sugar was determined separately in each fraction. In other experiments the animals were obtained, as before, immediately on arrival from the fish traps, from whence they were transported in a live car, brought to the laboratory, and placed in a large tank of running water. At the end of 20 to 30 minutes the animals, who by this time were somewhat, but apparently not entirely, recovered were rapidly removed from the tank, and

samples of blood taken at intervals as before. I have repeated this experiment many times with a number of different animals, but as the results were invariably uniform I present here the data of only two experiments, one on an animal in good condition, and one on a dogfish who had not entirely recovered from the rough handling and asphyxia incident to his capture.

From the results given in Table II it would seem that in an animal who had not been subjected to a preliminary period of asphyxia (by being carried from the live car to the laboratory) no change in the blood sugar can be noted during a period of asphyxia while blood was being taken. If on the other hand the animal was in poor condition before the experiment the blood

TABLE II.

Experiments Showing the Influence of Asphyxia on Blood Sugar in the Dogfish.

Amount of blood taken.	Time elapsed since fish were removed from water.	Blood sugar.	Remarks.
cc.	min.	mg. per 100 cc.	
2.2	0.5	180	Fish allowed to stay in laboratory
2.3	1.5	142	tank 20 minutes after being
2.8	2.5	105	carried from pier. Not yet
2.3	3.5	106	fully recovered.
2.3	0.5	125	Fish kept in laboratory tank for
23.5	4.5	125	32 hours before experiment. In
2.4	6.5	125	good condition.

sugar tends to be higher during the first 1 or 2 minutes and sinks after complete asphyxia and bleeding have been continued for several minutes. The latter finding is in accord with the experiment reported by Scott (10) on a shark in poor condition. On the whole it would seem that prolonged partial asphyxia has a marked effect on the blood sugar of fish, but that during the short period (2 to 4 minutes) usually occupied in desanguifying an animal no abnormal effects are to be noted. The blood sugar of the dogfish appears to be extremely variable, observations made on a considerable number of apparently normal animals have shown results varying from 80 to 181 mg. The great majority of these figures fall between 90 and 110 mg., which is about the level for normal human blood for the analytical method used. I have not obtained

any results confirmatory of the finding of Scott that occasionally dogfish are obtained whose blood is devoid of reducing bodies, the lowest figure in my series of results being 80 mg. per 100 cc. of blood.

The high level of amino nitrogen found in fish blood (usually about 28 mg., four to five times the values reported for human blood, in which fluid concentrations of from 5 to 7 mg. may be considered as normal amounts) has led me to make a few analyses of the amino-acid content of fish muscle in order to determine whether the same relation obtained between the amino-acids of blood and tissue as found in mammals.

TABLE III.

Determinations of Amino Nitrogen in the Blood and Muscles of Fish and Marine Invertebrates.

Name.	Amino N per 100 gm.	
	Blood.	Muscle.
	mg.	*mg.*
Dusky shark...................................	31	40
Sand shark.............	39	73
Dogfish.......................................	30	32
Cunner..	28	32
Limulus......................................	0	15.6
Lobster.	3.3	40

Muscle from the flank was removed immediately after the withdrawal of blood, freed from fragments of skin and fat, cut into small pieces, and 10 gm. portions were rapidly weighed out and ground for 10 minutes with 10 gm. of clean dry sand. To this mixture there were added 70 cc. of ice-cold water and after further grinding of the mixture of muscle, sand, and water there were added 10 cc. of 10 per cent sodium tungstate and 10 cc. of $\frac{2}{3}$ N sulfuric acid. After thorough stirring the mixture was allowed to stand for 10 to 15 minutes and then filtered. Amino nitrogen in the filtrate was then determined by the colorimetric method of Folin. Some of the results obtained are given in Table III.

From these figures it is evident that not only is the amino nitrogen content of fish blood much higher than in mammals but that a different equilibrium apparently exists between blood

and muscle in these two classes of animals. The amino nitrogen content of dog blood and muscle was found by Van Slyke and Meyer (11) to be in the neighborhood of 6 and 50 mg., respectively, although relatively wide variations are noted (especially for muscle) depending on diet, time of feeding, etc. In other words, the dog has approximately ten times more amino-acids in his muscle than in his blood; in the fish an entirely different relation appears to obtain, as here the blood frequently contains almost as much amino-acids as does the muscle.

In Table III are also included a few determinations on the blood and muscle of two species of invertebrates. These determinations were made because of the suggestion of Myers (12) that in all probability the blood of invertebrates contains large amounts of amino-acids. As will be seen the surmise of Myers was incorrect, as in several of these animals amino-acids appeared to be practically absent from the body fluids whereas considerable quantities still exist in the muscle. The lobsters examined were, however, an exception to this rule in that they showed small amounts of amino nitrogen in the blood and larger quantities in the muscle than did the other invertebrates. It is probable that this difference between the lobster and the horseshoe crab may in part at least be due to the state of nutrition of the animals, the lobsters having been examined within 24 hours after being taken whereas the *Limuli* had been in captivity for weeks and were probably distinctly undernourished.

SUMMARY.

Analyses of the blood of three species of elasmobranch and four species of teleost fish indicate that the values for the non-protein constituents of fish blood published in 1913 will, as the result of improved analytical technique, require revision.

The following may be said to represent average values for elasmobranch blood: Non-protein nitrogen 1,000; urea nitrogen 800; amino nitrogen 28; creatinine 6; creatine 25; uric acid 1.1 mg. For teleost blood the following are average figures: Non-protein nitrogen 65; urea nitrogen 9; amino nitrogen 28; creatinine 1.0; creatine 6; uric acid 4 mg. The blood of invertebrates frequently contains no amino nitrogen although considerable amounts of this fraction are demonstrated in the muscle.

BIBLIOGRAPHY.

1. Denis, W., *J. Biol. Chem.*, 1913–14, xvi, 389.
2. Folin, O., and Wu, H., *J. Biol. Chem.*, 1919, xxxviii, 81.
3. Folin, O., *J. Biol. Chem.*, 1922, li, 377.
4. Benedict, S. R., *J. Biol. Chem.*, 1922, li, 187.
5. Wilson, D. W., and Adolph, E. F., *J. Biol. Chem.*, 1917, xxix, 405.
6. Folin, O., *The Harvey Lectures*, 1920–21, xv, 109. Nash, T. P., Jr., and Benedict, S. R., *J. Biol. Chem.*, 1921, xlviii, 463.
7. Folin, O., and Denis, W., *J. Biol. Chem.*, 1916, xxvi, 491.
8. Folin, O., and Bell, R. D., *J. Biol. Chem.*, 1917, xxix, 329.
9. Lang, R. S., and Macleod, J. J. R., *Quart. J. Exp. Physiol.*, 1918–20, xii, 331.
10. Scott, E. L., *Am. J. Physiol.*, 1921, lv, 349.
11. Van Slyke, D. D., and Meyer, G. M., *J. Biol. Chem.*, 1913–14, xvi, 213.
12. Myers, R. G., *J. Biol. Chem.*, 1920, xli, 119.

THE BEHAVIOR OF CHLORIDES INTRODUCED INTO THE BLOOD UNDER NORMAL AND NEPHRITIC CONDITIONS.*

By FRANK P. UNDERHILL and EDWARD T. WAKEMAN.

(From the Department of Pharmacology and Toxicology, Yale University, New Haven.)

A study of chlorides in the body is becoming more and more important to a better understanding of nephritis, edema, and hypertension, and as a guide to the treatment of these conditions. Chlorides play an important rôle in determining the distribution of fluids in the body. A retention of chlorides in the blood has been noted in nephritis, particularly in cases with edema (1), and Allen (2) has endeavored to show that salt retention is the cause of pure hypertension and also of hypertension in many cases of kidney disease. Many authors consider chloride retention as the chief causative factor in edema. The edema develops when the threshold of excretion of chlorides is raised and the edema fluid is reabsorbed when this threshold returns to normal.

Höst (3) studied the chloride concentration in the blood of nine patients suffering from nephritis with and without edema and found the chlorides to be high while at the same time the chloride output was incomplete. He believed, therefore, that the cause of chloride retention in these cases of nephritis was mainly to be found in the kidneys which, to a certain extent, bar the way for chloride excretion. When this barrier gave way more or less suddenly, as apparently happened in two cases when the patients received a single large dose of salt, the retained chlorides and water rushed out. With improvement in these cases the chloride

---

* The data are taken from the thesis of Dr. Edward T. Wakeman presented in candidacy for the degree of Doctor of Medicine, Yale University, 1922.

excretion was increased and the blood chlorides returned to normal concentration.

On the other hand, Achard and Ribot (4) have observed some cases of nephritis in which there was a retention of chlorides without an increase in the concentration of chlorides in the blood. This retention cannot be satisfactorily explained by impaired kidney function and it seems more probable that the cause is extrarenal, in the capillaries or even in the tissues.

A considerable amount of experimental work has been done to study the changes in blood concentration in nephritis. Boycott (5) found that in normal rabbits the original volume of blood was restored in a short and fairly constant interval after intravenous injection of Ringer's solution and that the activity of the kidneys was not essential to this regulation of the blood volume. However, rabbits rendered nephritic by uranium nitrate showed a partial failure of the blood volume to return to normal under the same experimental procedure. Chisolm (6) found a similar incomplete regulation of the blood volume in rabbits with nephritis induced by potassium chromate; and Bogert, Underhill, and Mendel (7) have shown the same condition to be true in rabbits with nephritis induced by sodium tartrate. An alteration of the permeability of the blood vessel walls seems the only adequate explanation of the changed conditions prevailing in nephritic animals. Bogert, Underhill, and Mendel have concluded, therefore, that the condition of nephritis effects an alteration of the permeability of the walls of the capillaries of the general circulation in such a manner as to hinder the passage of fluid from the blood to the tissues.

In this connection it is interesting to note that Smith and Mendel (8), in studying the regulation of blood volume after the injection of solutions of various sodium salts, found that with citrate, tartrate, and sulfate solutions the blood volume stayed above normal longer than with any other salts. With chloride solution the return to normal was more rapid, while with bromide, acetate, and nitrate the return to normal was most rapid. They suggest that the citrate, tartrate, and sulfate salts may have decreased the permeability of the capillary membranes so that the free movement of surplus fluid out of the circulation observed with chloride, for instance, was interfered with. If this is true, is it not possible that the alteration in permeability of capillary walls noted

by Bogert, Underhill, and Mendel (7) was due, not to the nephritis but rather to the sodium tartrate used to produce the nephritis?

These studies have been concerned with the regulation of the blood volume after injections of saline solution in normal and nephritic rabbits with special reference to the fate of the injected fluid. In the following experiments it is proposed to study the regulation of the blood volume after injections of saline solution in normal and nephritic rabbits with special reference to the fate of the chlorides injected.

Methods.

In general, normal full grown rabbits which fasted 48 hours were used in all of the experiments. Hemoglobin was considered a true measure of the dilution of the blood as worked out by Bogert, Underhill, and Mendel (7) and Smith and Mendel (8) under similar experimental conditions. After the concentrations of chloride and hemoglobin in the blood were determined, a known quantity of salt solution was forced into the circulation as rapidly as possible. Immediately after injection and at regular intervals thereafter, the concentrations of chloride and of hemoglobin were measured and the relative blood volumes were calculated from the latter figures.

Technique.—Sodium chloride solution of exactly 0.9 per cent concentration, warmed to 38°C., was used for injection of chloride. This solution was introduced into the ear vein in rabbits under sufficient pressure so that 80 cc. were injected in 2 minutes. Samples of blood were taken from the vessels of the rabbit's ear, collecting the blood drop by drop into beakers containing a few crystals of potassium oxalate, 2 cc. being necessary for each chloride determination. The blood for hemoglobin determination was drawn up into a 20 mm. pipette directly from the bleeding ear vein at the same time that the blood was collected for chloride determination.

The concentration of chlorides was determined by the method of Whitehorn (9). Hemoglobin determinations were made according to the method of Cohen and Smith (10).

Experimental nephritis was induced by sodium tartrate according to the procedure suggested by Underhill, Wells, and Goldschmidt (11). The rabbits were given no food for 2 days prior to

receiving a subcutaneous injection of definite amounts of sodium tartrate, formed by dissolving a weighed amount of tartaric acid in water and neutralizing with concentrated sodium hydroxide to a faintly alkaline reaction.

Control Experiments.

Effect of Frequent Bleeding upon Chlorides and Hemoglobin.

In order to determine the effect of bleeding a rabbit, 2 cc. or more at a time, at the frequent intervals set down by these experi-

TABLE I.

Effect of Repeated Small Hemorrhages upon the Concentrations of Chlorides and Hemoglobin in the Blood.

Time.	Rabbit A. Weight = 2,350 gm.			Rabbit B. Weight = 2,000 gm.		
	Chloride as NaCl per 100 cc. blood.	Hemo-globin.	Relative blood volume in percentage of normal.	Chloride as NaCl per 100 cc. blood.	Hemo-globin.	Relative blood volume in percentage of normal.
		per cent			per cent	
Normal.	556	81	100		107	100
5 minutes.	553	82	99	456	91	118
10 "	542	80	101	456	101	106
15 "	556	71	114	459	89	120
30 "	539	75	108	451	93	115
60 "	553	72	113	459	100	107
2 hours.	553	70	116	462	97	110
3 "	561	69	117	481	92	116
4 "	600	62	131	512	89	120
5 "	572	61	133	528	85	126

ments, the chloride and hemoglobin concentrations of normal rabbits were determined at regular intervals. The results of two such experiments are given in Table I.

From these experiments it appears that, in normal fasting rabbits, hemorrhage, as practised in present experiments, may or may not significantly change the chloride content of the blood. When there is a significant dilution of the blood as the result of hemorrhage, the chlorides are correspondingly increased. The dilution of the blood and the increase in chlorides are more marked after the 1st or 2nd hour. As a rule there is a marked general

parallelism between the relative blood volume and the chloride content of the blood.

The Chloride and Hemoglobin Concentrations in Normal Rabbits during Fasting.

As a control upon the effect of sodium tartrate in the production of nephritis, the chloride and hemoglobin concentrations were determined twice a day on normal fasting rabbits for a period of 5 days. The results of two such experiments are given in Table II.

TABLE II.

Effect of Fasting upon the Concentrations of Chloride and Hemoglobin in the Blood.

Time.	Day of fasting.	Rabbit C.			Rabbit D.		
Day.	Hour.	Chloride as NaCl per 100 cc. blood.	Hemo-globin.	Relative blood volume in percentage of normal.	Chloride as NaCl per 100 cc. blood.	Hemo-globin.	Relative blood volume in percentage of normal.
			per cent			*per cent*	
3rd	9.00 a.m.	571	81	100	514	92	100
	5.00 p.m.	571	78	104	501	82	112
4th	9.00 a.m.	583	77	105	465	78	118
	5.00 p.m.	566	74	110	476	74	124
5th	9.00 a.m.	564	75	108	489	71	130
	5.00 p.m.	559	74	110	489	69	133
6th	9.00 a.m.	566	64	127	512	73	126
	5.00 p.m.	553	65	125	511	70	131
7th	9.00 a.m.	561	66	123	542	64	144

From these experiments it appears that the chloride concentration of the blood remains practically constant or may be slightly increased during fasting when normal rabbits are bled twice a day for several days. The relative blood volume, however, increases progressively during this period.

Nephritic Rabbits during Fasting.

Effect of a Sublethal Dose of Sodium Tartrate on the Chloride and Hemoglobin Concentration of a Normal Fasting Rabbit.

A normal fasting rabbit, weighing 2,260 gm., was given a subcutaneous injection of tartaric acid, 0.75 gm. per kilo of body

weight, neutralized with sodium hydroxide. The chloride and hemoglobin concentrations were determined before injection of tartaric acid and twice daily thereafter for 5 days. The rabbit drank freely, but 21 hours after injection became anuric and remained so until the 3rd day, after which it excreted a small quantity of urine each day. The rabbit was extremely weak on the 3rd day and lay on its side in a semicomatose condition. On the 4th day it seemed stronger and was more active and seemed

TABLE III.

Effect of a Sublethal Dose of Sodium Tartrate on the Chloride and Hemoglobin Concentrations of a Normal Fasting Rabbit.

Time. Day of fasting.		Rabbit F.　Weight = 2,260 gm.			
Day.	Hour.	Chloride as NaCl per 100 cc. blood.	Hemoglobin.	Relative blood volume in percentage of normal.	Excretion of urine.
			per cent		*cc.*
3rd	4.00 p.m.	509	89	100	130
	6.00 "	Injected 1.7 gm. tartaric acid.			
4th	9.00 a.m.	484	61	110	90
	5.00 p.m.	415	70	127	0
5th	9.00 a.m.	360	67	132	0
	5.00 p.m.	352	73	122	0
6th	9.00 a.m.	344	64	139	65
	5.00 p.m.	352	61	145	36
7th	9.00 a.m.	375	61	145	60
8th	9.00 "	429	66	135	160
9th	9.00 "	481	63	141	66

to improve each day until it died on the 8th day after injection. The results of this experiment are given in Table III.

In the single representative of this type of experiment it will be seen that there is a marked drop in chloride concentration during the period of severe nephritis as shown by anuria. This is in striking contrast to the more or less constant chloride concentration in a normal rabbit under similar experimental conditions as shown in Table II. At the same time the relative blood volume shows a corresponding increase, more rapid than that observed in normal rabbits under similar conditions. Later, however, as there is an apparent change toward recovery as shown by excretion of urine, the relation of chloride concentration to relative blood volume

changes. The relative blood volume remains more or less stationary, whereas the chloride concentration tends to regain its normal level.

Effect of Fatal Doses of Sodium Tartrate on the Chloride and Hemoglobin Concentrations of Normal Fasting Rabbits.

Normal fasting rabbits were given subcutaneous injections of tartaric acid, 1 gm. per kilo of body weight, neutralized with sodium hydroxide. The chloride and hemoglobin concentrations were determined before injection and twice daily thereafter until

TABLE IV.

Effect of Fatal Doses of Sodium Tartrate on the Chloride and Hemoglobin Concentrations of Normal Fasting Rabbits.

Time.	Day of fasting.	Rabbit K. Weight = 2,360 gm.			Rabbit M. Weight = 2,200 gm.		
Day.	Hour.	Chloride as NaCl per 100 cc. blood.	Hemo-globin.	Relative blood volume in per-centage of normal.	Chloride as NaCl per 100 cc blood.	Hemo-globin.	Relative blood volume in per-centage of normal
		per cent			*per cent*		
3rd	9 00 a.m.	506	86	100	556	77	100
	10.30 "	Injected 2.4 gm. tartaric acid.			Injected 2.2 gm. tartaric acid.		
	4.30 p.m.	523	84	102			
4th	9.00 a.m.	446	78	110	522	67	115
	4.30 p.m.	415	83	104			
		Died on 4th day after injection.			Died on 2nd day after injection.		

the animals died. These rabbits drank freely, but became completely anuric on the day following injection. Of three such rabbits one died 2 days, one 3 days, and one 4 days after injection. The results of these experiments are given in Tables IV and V.

In these, as in the previous experiments, it will be seen that there is a more or less rapid decrease in chloride concentration after the induction of tartrate nephritis and an increase in the relative blood volume. The dilution of the blood in tartrate nephritis confirms the observations of Underhill and Greenhouse (12) on this condition.

TABLE V.

Effect of a Fatal Dose of Sodium Tartrate on the Chloride and Hemoglobin Concentration of a Normal Fasting Rabbit.

Time. Day of fasting.		Rabbit I. Weight = 3,000 gm.			
Day.	Hour.	Chloride as NaCl per 100 cc. blood.	Hemo-globin.	Relative blood volume in per-centage of normal.	Excretion of urine.
			per cent		*cc.*
3rd	4.30 p.m.	514	82	100	150
	5.00 "	Injected 3 gm. tartaric acid.			
4th	9.00 a.m.	435	99	91	0
	4.30 p.m.	462	77	107	Few drops expressed.
5th	9.00 a.m.	451	75	109	0
	4.30 p.m.	440	63	131	0
6th	9.00 a.m.	451	53	156	0
	4.30 p.m.	437	48	170	Few drops expressed.
7th	9.00 a.m.	484	32	254	0
	4.30 p.m.	484	28	292	0
		Found dead on 5th day after injection.			

Experiments with Injection of Salt Solution.

Influence of the Intravenous Injection of Isotonic Sodium Chloride Solution upon Sodium Chloride Content of the Blood and upon the Relative Blood Volume of Normal Fasting Rabbits.

The chloride and hemoglobin concentrations of the blood were determined before the injection of salt solution. Then 80 cc. of 0.9 per cent NaCl solution were introduced into the ear vein of normal fasting rabbits under sufficient pressure to inject the entire amount in 2 minutes. The chloride and hemoglobin concentrations were determined immediately and at regular intervals thereafter. The results of two such experiments are given in Table VI.

It will be seen from these experiments that there is at first a rapid rise in the chloride content of the blood which is closely paralleled by the relative blood volume. In general the chloride content returns to a level near normal or even to normal within a relatively short time — 30 minutes. However, the normal level may not be maintained, probably because of the fact that the

hemorrhage effect observed previously now comes into play. This is especially noticeable after a period of an hour or more. The relative blood volume follows closely the changes in the chloride content of the blood.

TABLE VI.

Influence of Intravenous Injection of Isotonic Sodium Chloride Solution upon the Chloride Content of the Blood and upon the Relative Blood Volume.

Time.	Rabbit N. Weight = 2,200 gm.			Rabbit P. Weight = 3,100 gm.		
	Chloride as NaCl per 100 cc. blood.	Hemo-globin.	Relative blood volume in per-centage of normal.	Chloride as NaCl per 100 cc. blood.	Hemo-globin.	Relative blood volume in per-centage of normal.
		per cent			per cent	
Before injection......	520	80	100	494	102	100
After injection.						
Immediately.....	586	76	105	556	76	134
5 minutes.......	558	70	115*	528	79	129
10 "	547	73	110	495	87	117
15 "	545	72	111	514	89	115
30 "	550	77	104	509	92	111
1 hour..........	558	75	107	536	83	123
2 hours..........	575	75	107	542	84	121
3 "	578	66	121			
4 "	575	64	125	567	79	129
5 "	569	56	143	564	77	132
6 "	586	60	134	591	75	136

* In the subsequent analysis of this table, this figure was taken instead of 105 as representing the more probable volume immediately after injection.

Influence of the Intravenous Injection of Isotonic Sodium Chloride Solution upon the Chloride Content of the Blood and upon the Relative Blood Volume of Nephritic Rabbits.

Normal fasting rabbits were rendered nephritic by the sub-cutaneous injection of 1 gm. of tartaric acid per kilo of body weight, neutralized with sodium hydroxide. Since rabbits thus treated are completely anuric the day following injection, and may die on the 2nd day, they were used for experimentation 24 hours after tartrate injection. The same procedure was followed as described for normal rabbits in determining the effect of fatal doses of sodium

tartrate on the chloride and hemoglobin concentrations. The results of two such experiments are given in Table VII.

It will be seen from these results that the immediate response to sodium chloride injections into nephritic rabbits varies but little from that observed with normal animals. That is to say, the relative blood volume and chloride content of the blood follow curves very similar in their time relations to those obtained with normal fasting animals under similar experimental conditions.

TABLE VII.

Influence of the Intravenous Injection of Isotonic Sodium Chloride Solution upon the Chloride Content of the Blood and upon the Relative Blood Volume of Nephritic Rabbits.

Time.	Rabbit R. Weight = 2,700 gm.			Rabbit S. Weight = 2,450 gm.		
	Chloride as NaCl per 100 cc. blood.	Hemo-globin.	Relative blood volume in percentage of normal.	Chloride as NaCl per 100 cc. blood.	Hemo-globin.	Relative blood volume in percentage of normal.
		per cent			*per cent*	
Before injection......	448	76	100	507	93	100
After injection.						
Immediately.....	558	61	125	550	65	143
5 minutes.......	517	64	119	550	74	126
10 "	514	69	110	542	79	118
15 "				525	79	118
30 "	476	70	109	523	83	112
1 hour..........	501	76	100	547	72	129
2 hours.........	476	62	123	536	71	131
3 "	501	62	123	545	71	131
4 "				523	71	131
5 "	503	58	131	539	70	133
6 "	503	53	143	550	61	153

From this one may perhaps infer that, in this type of nephritis at least, the capacity of the organism to adjust itself to alterations in fluid content of the blood is not impaired as judged by the criteria adopted. On the other hand, in the later time intervals studied, it would appear that chlorides leave the tissues and enter the blood stream less readily than is true for normal animals. May one infer from this that a change has occurred in the capillary wall of such a nature that absorption is not hindered from the blood to the tissues but that in the opposite direction a barrier is

interposed, or may one assume that the tissues themselves have a greater affinity for sodium chloride?

In order to compare the results of the last two experiments more accurately, an analysis was made of the figures obtained with special reference to the amount of injected fluid and of injected NaCl which had passed out of the circulation at any given time. For the purpose of calculating the absolute blood volume it was assumed that the average rabbit of 2 to 3 kilos has 50 cc. of blood per kilo of body weight (5).

With Rabbit N therefore, in Table VI, the assumed volume would be $\frac{2,200}{1,000} \times 50 = 110$ cc. 80 cc. of salt solution were introduced in 2 minutes. If the entire amount of injected liquid had still been in the circulation at the end of the injection period, the volume of blood would have been 110 + 80 = 190 cc. and the hemoglobin would have been $\frac{110}{190} \times 80$ per cent = 46 per cent. Immediately on completion of the injection, however, the hemoglobin was found to be 70 per cent, giving a relative blood volume of $\frac{80}{70} \times 100 = 115$ instead of $\frac{80}{46} \times 100 = 174$, and an absolute volume of $\frac{110}{100} \times 115 = 127$ cc. instead of 190 cc. Evidently, therefore, 190 − 127 = 63 cc. or 79 per cent of liquid injected had passed out of the vessels during the period of injection.

Before injection of salt solution, Rabbit N had 520 mg. of NaCl per 100 cc. of blood. If the absolute blood volume was 110 cc., as assumed above, the total chloride content of the blood would have been $\frac{110}{100} \times 520 = 572$ mg. of NaCl. The injected salt solution was exactly 0.9 per cent NaCl. Hence, $80 \times 9.0 = 720$ mg. of NaCl were injected. If the whole amount of injected salt had still been in the circulation at the end of the injection period, the total chloride content of the blood would have been 572 + 720 = 1,292 mg. of NaCl. Immediately on completion of injection, however, the chloride content of the blood was 586 mg. of NaCl per 100 cc. The absolute blood volume, as determined above from the relative blood volume immediately after injection, was 127 cc. The total amount of chloride in circulation, then, was $586 \times \frac{127}{100} = 744$ mg. of NaCl instead of 1,292 mg. of NaCl. Evidently, therefore, 1,292 − 744 = 548 mg. of NaCl or 76 per cent of the chloride injected has passed out of the vessels during the period of injection.

Similar calculations were made for the normal Rabbit P and for the two nephritic rabbits, Rabbits R and S. The percentage of

Chlorides Introduced into Blood

injected fluid and also of injected NaCl which had passed out of the circulation at any given time is represented in Table VIII.

There are several sources of error in these figures. First there is room for considerable error in collecting the blood drop by drop from an ear vein. The blood may flow freely and a 2 cc. sample be collected in a minute's time. On the other hand, it may take 5 minutes to collect a sample. The error from this variation would be most marked in the first three or four determinations after injection of the salt solution. After half an hour such variation is of minor importance.

TABLE VIII.

Percentage of Injected Liquid and Injected Sodium Chloride Which Has Passed out of the Circulation at Any Given Time Following the Injection.

	Percentage of injected liquid lost. Time after injection.											Percentage of injected chloride lost. Time after injection.										
	Minutes.					Hours.						Minutes.					Hours.					
	0	5	10	15	30	1	2	3	4	5	6	0	5	10	15	30	1	2	3	4	5	6
Rabbit N	79	79	86	85	95	90	90	71				65	41	54	76	81	88	87	92	88	85	69
" P	34	44	68	71	79	55	59					44	38	30	46	60	82	79	85	64	65	49
Average	57	62	77	78	87	73	75					55	40	42	61	71	85	83	89	76	75	59
Rabbit R	76	69	83		86	100	61	61				48	28	53	69	78	88	90	107	68	60	49
" S	34	60	73	73	81	55	52	52				52	49	19	52	68	77	81	86	65	66	64
Average	55	65	78		84	78	57	57				49	24	53	69	78	87	78	87	66	62	46

Secondly, the hemorrhage factor has not been considered. 20 to 25 cc. of blood were withdrawn in the course of each experiment. However, this fact was the same in each experiment, so the results are comparable.

Thirdly, one is not justified in drawing any definite conclusions from the results of so few experiments. As will be seen in comparing Rabbit N with Rabbit P, or Rabbit R with Rabbit S, there is a large variation between animals of the same type of experiment.

Recognizing the inaccuracy of these results it is nevertheless interesting to compare the averages of each type of experiment—the injection of salt solution into normal rabbits and into nephritic rabbits. There is a striking parallelism between the normal and nephritic rabbits, both in the passage of fluid and of chlorides

from the vessels into the tissues and from the tissues into the blood stream, and in the relation of NaCl to fluid.   During the first half hour the greater part, 80 to 90 per cent, of the fluid injected and also of the NaCl injected passes out of the circulation.   There is apparently no difference between normal and nephritic rabbits in this respect.   After the first half hour, fluid and NaCl pass into the circulation, and the chloride content of the blood and relative blood volume are increased.   This is probably due, in part at least, to the hemorrhage effect observed previously.   It would also appear from these figures that chlorides pass less readily from the tissues into the blood stream than the fluid, and there is very little difference between normal and nephritic rabbits in this respect.

DISCUSSION OF RESULTS.

The results of these experiments show a rather close parallelism between the behavior of the injected NaCl and the injected fluid. That is, in normal rabbits the blood volume tends to return to normal in a relatively short time, about 30 minutes, after the injection of isotonic salt solution.   The chloride content of the blood also tends to return to normal in the same short interval of time after the injection of sodium chloride in solution.   In nephritic animals, on the other hand, there is a partial failure of the blood volume to return to normal and this also appears to be true of the chloride content of the blood.   It would seem, therefore, that the barrier to the complete restoration of blood volume in nephritic animals also prevents the complete restoration of the chloride content of the blood.   An alteration of the permeability of the blood vessel walls seems the only adequate explanation of the changed condition prevailing in rabbits rendered nephritic by sodium tartrate.   It is therefore concluded that the condition of nephritis induced by sodium tartrate effects an alteration of the permeability of the walls of the capillaries of the general circulation in such a way as to hinder the passage of fluid and of NaCl from the blood to the tissues.

CONCLUSIONS.

In normal fasting rabbits, hemorrhage, as practised in the present experiments, may or may not significantly change the chloride

content of the blood. When there is a significant dilution of the blood as the result of hemorrhage, the chlorides are correspondingly increased. As a rule there is a marked general parallelism between the relative blood volume and the chloride content of the blood.

During fasting the chloride concentration of the blood remains practically constant or may be slightly increased when normal rabbits are bled daily, as practised in these experiments. The relative blood volume increases progressively under the same conditions.

In severe nephritis produced by a sublethal dose of sodium tartrate there is a marked decrease in the chloride concentration of the blood and a corresponding increase in the relative blood volume. As there is an apparent change toward recovery the chloride concentration of the blood increases and tends to regain its normal level, whereas the relative blood volume remains more or less stationary.

In fatal tartrate nephritis there is a more or less rapid decrease in the chloride concentration of the blood and an increase in the relative blood volume.

In normal fasting rabbits the intravenous injection of isotonic sodium chloride solution is followed by a rapid increase in the chloride content of the blood and a corresponding increase in the relative blood volume. The chloride content of the blood tends to return to normal within a relatively short time—30 minutes. However, the normal level may not be maintained, probably because of the fact that the hemorrhage effect observed previously now comes into play. The relative blood volume follows closely the changes in the chloride content of the blood.

In nephritic rabbits the intravenous injection of isotonic sodium chloride solution is also followed by a rapid increase in the chloride content of the blood and a corresponding increase in relative blood volume, and the chloride content and relative blood volume tend to return to normal in a relatively short time. After an hour or more, however, the chlorides tend to leave the tissues and enter the blood stream less readily in nephritic rabbits than is true for normal animals.

BIBLIOGRAPHY.

1. Widal, F., Ambard, L., and Weill, A., *Semaine méd.*, 1912, xxxii. 361.
2. Allen, F. M., *J. Am. Med. Assn.*, 1920, lxxiv, 652.
3. Höst, H. F., *J. Lab. and Clin. Med.*, 1919–20, v, 713.
4. Achard, C., and Ribot, A., *Semaine méd.*, 1913, xxxiii, 409.
5. Boycott, A. E., *J. Path. and Bact.*, 1913–14, xviii, 11.
6. Chisolm, R. A., *J. Path. and Bact.*, 1913–14, xviii, 552.
7. Bogert, L. J., Underhill, F. P., and Mendel, L. B., *Am. J. Physiol.*, 1916, xli, 189.
8. Smith, A. H., and Mendel, L. B., *Am. J. Physiol.*, 1920, liii, 323.
9. Whitehorn, J. C., *J. Biol. Chem.*, 1920–21, xlv, 449.
10. Cohen, B., and Smith, A. H., *J. Biol. Chem.*, 1919, xxxix, 489.
11. Underhill, F. P., Wells, H. G., and Goldschmidt, S., *J. Exp. Med.*, 1913, xviii, 322.
12. Underhill, F. P., and Greenhouse, B., *J. Urol.*, 1921, vi, 83.

THE INFLUENCE OF SUBCUTANEOUS INJECTIONS OF INDOLE AND SKATOLE UPON THE NITROGENOUS METABOLISM OF THE RABBIT.

By FRANK P. UNDERHILL and ROBERT KAPSINOW.

(*From the Department of Pharmacology and Toxicology, Yale University, New Haven.*)

Much interest is manifested in the possible detrimental effects of the products of intestinal putrefaction formed in such conditions as chronic constipation, intestinal obstruction, etc. The isolation and testing of the substances assumed to be responsible for many of the symptoms characteristic of these abnormal states have not been extensively undertaken for obvious reasons. Although indole and skatole are at once called to mind when intestinal putrefaction is under consideration the agency of these substances in the production of untoward symptoms has not been clearly demonstrated. It is true that Herter[1] has shown that indole produces depression of the circulation and respiration together with clonic convulsions. In man symptoms of neurasthenia with fatigue, depression, and insomnia are prominent when an abnormally large amount of indole is absorbed either as such experimentally or as a result of increased intestinal putrefaction. Doses of 2 gm. of indole per kilo subcutaneously introduced into rabbits produce death in a relatively short period.

The following investigation was planned to determine the influence of subcutaneous injections of indole and skatole upon nitrogenous metabolism in the rabbit.

Methods.

Normal full grown rabbits were fasted 2 days previous to the injection of indole and skatole. Distilled water was allowed.

---

[1] Herter, C. A., *New York Med. J.*, 1898, lxviii, 89.

Urine was collected in 24 hour periods. At the beginning of the 3rd day 30 mg. of indole per kilo were dissolved in $2\frac{1}{2}$ cc. of ether and injected subcutaneously. The same dosage and procedure were employed for skatole. Urinary analyses included total nitrogen, creatine, creatinine, total sulfates, and inorganic and ethereal sulfates by the usual Folin methods. Observations were continued for 2 days subsequent to injection. As a solvent ether was employed instead of acetone, as used by Salant and Kleitman.[2] Acetone is very toxic, whereas ether may be given subcutaneously in doses up to 5 cc. without influence upon nitrogenous metabolism.

The Influence of Indole and Skatole upon Nitrogenous Metabolism.

The experiments were divided into two groups: (a) controls; and (b) experiments testing the influence of indole and skatole. In Tables I and II are data derived from the control experiments.

TABLE I.

The Influence of Fasting upon Urinary Composition.

Rabbit A 1.   Control.   Fasting.

Date.	H₂O intake.	Urine.	Specific gravity	Reaction.	Total N.	Creatinine.	Creatine.	Inorganic sulfates.	Ethereal sulfates.	Total sulfates.
1921	*cc.*	*cc.*			*gm.*	*mg.*	*mg.*	*mg.*		*mg.*
Aug. 21	400	330	1.005	Alkaline.	0.210	50.4	9.2	44.1	—	10.9
" 22	220	120	1.007	Acid.	0.353	48.8	19.2	96.0	—	21.2
" 23	80	90	1.012	"	0.675	52.9	71.7	20.0	—	22.4
" 24	100	80	1.010	"	0.728	34.7	89.9	20.8	—	38.8

It may be stated that in order to save space only samples of the experiments are given; other protocols are omitted. However, all the data obtained are in agreement. In Tables III and IV results with indole may be found. In Tables V and VI the skatole experiments are detailed.

[2] Salant, W., and Kleitman, N., *J. Pharmacol. and Exp. Therap.*, 1922, xix, 307.

Comparison of the data of the controls and of the experimental animals shows that indole and skatole are without influence upon nitrogenous metabolism when introduced subcutaneously in a

TABLE II.

The Influence of Fasting upon Urinary Composition.

Rabbit A 3.   Control.   Fasting.

Date.	H₂O intake.	Urine.	Specific gravity.	Reaction.	Total N.	Creatinine.	Creatine.	Inorganic sulfates.	Ethereal sulfates.	Total sulfates.
1921	*cc.*	*cc.*			*gm.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
Sept. 11	300	330	1.010	Acid.	0.588	49.0	33.2	32.1	10.0	53.2
" 12	290	250	1.012	"	0.602	72.5	Lost.	4.9	9.2	18.0
" 13	250	220	1.008	"	0.828	71.5	43.2	50.2	8.3	49.7
" 14	296	340	1.008	"	0.378	69.4	22.0	57.5	8.1	Lost.

TABLE III.

The Influence of Indole upon Urinary Composition.

Rabbit A 4.   Indole.   Fasting.   Weight 2,400 gm.

Date.	H₂O intake.	Urine.	Specific gravity.	Reaction.	Total N.	Creatinine.	Creatine.	Inorganic sulfates.	Ethereal sulfates.	Total sulfates.	Indican test.
1921	*cc.*	*cc.*			*gm.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	
Sept. 19	50	52	1.020	Acid.	0.510	66.9	10.9	13.7	9.0	Lost.	0
" 20*	45	40	1.030	"	0.585	68.0	10.6	38.4	13.0	59.6	0
" 21	60	20	1.032	"	0.585	65.9	11.9	39.1	9.8	58.6	0
" 22	20	26	1.031	"	0.750	65.3	14.1	36.7	8.9	44.9	+++

* Injected.   Dose 15 mg. per kilo.   Total dosage, 36 mg. in 2 cc. of ether.

single dose. Indole appears to increase somewhat the excretion of ethereal sulfates which, however, was not true for skatole. In other respects the urinary composition as tested varied within the limits set by the controls. The introduction of these substances in the doses given failed to call forth any noteworthy symptoms.

TABLE IV.

The Influence of Indole upon Urinary Composition.

Rabbit A 4.   Indole.   Fasting.   Weight 2,300 gm.

Date.	H₂O intake.	Urine.	Specific gravity.	Reaction.	Total N.	Creatinine.	Creatine.	Inorganic sulfates.	Ethereal sulfates.	Total sulfates.	Indican test.
1921	cc.	cc.			gm.	mg.	mg.	mg.	mg.	mg.	
Sept. 26	205	84	1.010	Acid.	0.420	68.7	3.5	11.2	7.3	25.2	0
" 27*	50	34	1.020	"	0.568	61.6	7.4	9.8	8.1	28.0	±
" 28	65	64	1.010	"	0.798	65.5	10.5	8.4	19.0	44.8	++++
" 29	90	78	1.010	"	0.840	64.1	13.0	7.0	6.2	25.2	+

* Injected. Dose, 30 mg. per kilo. Total dosage, 69 mg. in 2.5 cc. of ether.

TABLE V.

The Influence of Skatole upon Urinary Composition.

Rabbit B 2.   Skatole.   Fasting.   Weight 2,070 gm.

Date.	H₂O intake.	Urine.	Specific gravity.	Reaction.	Total N.	Creatinine.	Creatine.	Inorganic sulfates.	Ethereal sulfates.	Total sulfates.	Indican test.
1921	cc.	cc.			gm.	mg.	mg.	mg.	mg.	mg.	
Nov. 9	220	180	1.006	Acid.	0.660	87.0	39.4	7.0	12.0	29.8	0
" 10*	340	260	1.008	"	0.882	79.8	55.2	31.6	12.3	68.6	0
" 11	100	80	1.020	"	0.975	85.2	37.2	47.6	16.8	81.0	0
" 12	150	50	1.028	"	0.975	46.8	88.2	55.5	10.4	84.0	0

* Injected. Dose, 30 mg. per kilo. Total dosage, 62 mg. in 3 cc. of ether.

TABLE VI.

The Influence of Skatole upon Urinary Composition.

Rabbit B 3.   Skatole.   Fasting.   Weight 2,070 gm.

Date.	H₂O intake.	Urine.	Specific gravity.	Reaction.	Total N.	Creatinine.	Creatine.	Inorganic sulfates.	Ethereal sulfates.	Total sulfates.	Indican test.
1921	cc.	cc.			gm.	mg.	mg.	mg.	mg.	mg.	
Nov. 15	220	110	1.014	Acid.	1.170	108.0	11.1	112.0	Lost.	113.0	0
" 16*	110	100	1.018	"	0.950	101.2	23.6	84.0	10.2	139.0	0
" 17	110	70	1.020	"	0.870	98.7	48.7	77.0	9.4	73.0	0
" 18	100	86	1.014	"	0.945	95.3	17.2	86.0	9.2	97.0	0

* Injected. Dose, 30 mg. per kilo. Total dosage, 62 mg. in 3 cc. of ether.

THE PREPARATION OF NUCLEIC ACID FROM THE NUCLEOPROTEIN OF TUBERCLE BACILLI (TUBERCULINIC ACID).*

By TREAT B. JOHNSON and ELMER B. BROWN.

(From the Department of Chemistry, Yale University, New Haven.)

While it has been known for a long time that nucleoprotein constitutes a considerable part of the cell of tubercle bacillus it was not until 1898 that an attempt was made to separate a nucleic acid from this organism. This was undertaken by Ruppel,[1] who subjected large quantities of crushed bacilli to aqueous extraction and treatment with acetic acid and, succeeded in precipitating from the acid solution with hydrochloric acid and alcohol a product containing 9.42 per cent of phosphorus and possessing all the properties of a nucleic acid. Ruppel named this characteristic product "Tuberculinsäure", and described it as a substance possessing poisonous and immunizing properties, but beyond its similarity in properties to other nucleic acids and its high phosphorus content no further proof was given by him to establish its identity.

Ruppel's investigation was followed by the work of Levene[2,3] who succeeded in extracting from defatted and crushed tubercle bacilli with 5 per cent aqueous sodium chloride or 8 per cent ammonium chloride a small amount of phosphorus containing material possessing the properties of a nucleic acid. This product was never obtained, however, in pure condition, but that he was dealing with a nucleic acid was proved by the fact that it contained

---

[1] Ruppel, W. G., *Z. physiol. Chem.*, 1898–99, xxvi, 218.
[2] Levene, P. A., *J. Med. Research*, 1901, vi, 135.
[3] Levene, P. A., *J. Med. Research*, 1904, xii, 251.

phosphorus and gave on hydrolysis the two characteristic purines —guanine and adenine. Levene not only isolated free nucleic acid from tubercle bacilli by extraction with the above inorganic salt solutions, but he also subjected his bacilli residues to hydrolysis with 5 per cent sodium hydroxide solution and succeeded in this manner in freeing combined nucleic acid from the nucleoprotein constituents of the cell. This acid was separated, and subjected to acid hydrolysis when the two purines, guanine and adenine, were obtained. Levene was unable to establish from the results of his analyses that this acid from tubercle bacillus is identical with yeast or thymus nucleic acid.

Besides these two investigators no one has succeeded apparently in isolating any product from tubercle bacillus exhibiting the characteristic properties of a nucleic acid with the exception of the Japanese investigator Kitajima.[4] He succeeded in separating from this organism by an extraction process a poisonous constituent to which he gave the name "Tuberkelothymine Säure." While the composition of this acid was not determined, Kitajima states in his paper that the substance behaved in every way like thymus nucleic acid and yielded thymine or a similar body on hydrolysis.

In all the records of these researches no data are given indicating the yield of nucleic acid obtained from tubercle bacillus. Several investigators in this field have used the phosphorus content as an indirect measure of nucleic acid and calculated the amount of acid and nucleoprotein present on that basis, but it is now known that such calculations are inaccurate as the tubercle bacilli contain a large proportion of their phosphorus in other forms of organic combinations as phosphatides, lecithin, etc. The nitrogen hydrolytic products of nucleic acid such as the purines and pyrimidines are characteristic of these biochemical substances, and Long[5] has recently determined from the quantity of purines found after hydrolysis that the real nucleic acid content of defatted bacillus is between 2.0 and 3.5 per cent of the weight of this organism. The accuracy of this figure depends, of course, upon efficient removal of foreign material and thorough drying of the bacilli. The results obtained by Long, though lower than expected, represent very probably the best estimate

[4] Kitajima, T., *Mitt. Med. Ges. Tokio*, 1902, xvi. 17.

[5] Long, E. R., *Am. Rev. Tuberc.*, 1920–21, iv. 842.

of the actual nucleic acid content of tubercle bacillus which has thus far been made. A quantitative determination of the pyrimidine content of tubercle bacillus, which is equally characteristic of nucleic acids, has never been made.

The nucleic acid of tubercle bacillus was of interest to the writers not because of the supposed poisonous or immunizing properties ascribed to it by earlier investigators, but from the more fundamental standpoint of classification of the tubercle bacilli. The experimental evidence, thus far presented, indicates that the nucleic acids of plant and animal origin are quite different in chemical constitution, but so far as the writers are aware, no one has completely analyzed the nucleic acid of tubercle bacillus to ascertain the group to which this organism is to be assigned. Fortunately a sufficient quantity of this organism to undertake the first stages of such an investigation was placed at our disposal this past year, and in this paper we desire to render a preliminary and condensed report of certain results of our research. We will now present not only several new facts of interest bearing on the question of composition of tubercle bacilli, but also describe a method of operating whereby the nucleic acid of this unicellular organism can be obtained for experimental work without great difficulty.[6]

EXPERIMENTAL PART.

Supply of Tubercle Bacilli.

The tubercle bacilli which were furnished for our research through the courtesy of Parke, Davis and Co. consisted of human

bacilli and were received by us in the form of a heavy, light grey, granular powder resembling sand. The tubercle bacilli furnished by the Mulford Co. were a mixture of human and bovine bacilli and somewhat less granular than the Parke, Davis and Co. product, and much darker in color. The material supplied by both concerns had been well dried before shipment to our laboratory, the Parke, Davis and Co. and the Mulford Co. products losing only 1 and 1.5 per cent of water, respectively, after thorough grinding and drying *in vacuo* over concentrated sulfuric acid. After this treatment neither product lost further weight by heating to 110°C. at ordinary pressure or at 55°C. in a partial vacuum. The amount of tubercle bacilli furnished was 363 gm. of the human bacilli and 443 gm. of the mixed human-bovine bacilli or a total of 806 gm.

Fat Content of Tubercle Bacilli.

The solvent which we found most practical for the extraction of fat in our tubercle bacilli was anhydrous toluene, and it was our experience that the operation could be carried out efficiently and rapidly in large units by operating in an ordinary flask and decanting the toluene extract after digestion to dissolve fat. To avoid loss of bacilli by decantation of toluene the flask was connected to a reflux condenser and the toluene drawn off through a syphon tube after thorough settling. The extraction was hastened by heating with toluene at 90–100°C. for 7 to 8 hours and the solution then decanted after cooling. After repetition of this procedure five to six times with liberal quantities of toluene the extraction of fat was practically complete and the bacilli could be drained on a Buchner funnel with ease and then dried to constant weight by heating at 80°C. in a partial vacuum. From 361 gm. of Parke, Davis and Co. bacilli we obtained 236.3 gm. of defatted material, showing a loss in weight of 124.7 gm. (34.55 per cent). 425 gm. of the Mulford Co. bacilli were subjected to a similar extraction process in which we used 7 liters of anhydrous toluene. Here the loss in weight was actually 43.53 per cent; the defatted bacilli weighing 240 gm. after drying in a vacuum at 80°C. It is possible that some colloidal material besides fat was removed by the toluene, but nothing could be separated by filtering or centrifuging the toluene solution. These toluene extracts have been set aside for further research.

Analysis of Tubercle Bacilli for Inorganic Constituents.

The analytical values for ash are somewhat higher than those reported by De Schweinitz and Dorset,[7] and also by the Royal Commission,[8] but lower than the values published by Levene.[2] Goris[9] reports an ash content of 2.5 per cent in dried bacilli. Mention should also be made here of the fact that the ash of the original Mulford Co. bacilli gave on analysis a SO_4 content of 10.41 per cent, and also contained SiO_2 to the extent of 1.2 per cent. Since we did not observe the presence of silica in the Parke, Davis and Co. bacilli, we infer that this probably is an impurity in the Mulford Co. product and is not a constituent of normal tubercle bacilli. In general, the analytical values for inorganic

TABLE 1.

Sample.	Ash.	P_2O_5 in ash.	P_2O_5 in bacilli.	CaO in ash.	CaO in bacilli.	MgO in ash.	MgO in bacilli.
	per cent	per cent	per cent	per cent	per cent	per cent	per cent
Parke, Davis and Co. original bacilli.............	4.19	47.88	2.01				
Parke, Davis and Co. bacilli after toluene extraction..	5.44	46.12	2.49				
Mulford Co. original bacilli,	5.78	39.66	2.29	7.16	0.41	8.52	0.47
Mulford Co. bacilli after toluene extraction.......	9.41 9.2	17.60	1.65	1.16		0.68	

constituents agree quite well with those obtained by previous investigators, and the results tend to confirm the conclusions already made that no definite distinction between viruses of different origin can be established by these analytical values. The composition of the ash of tubercle bacilli is dependent apparently upon the conditions influencing the organisms during their growth.

Inorganic Constituents Extracted by Toluene.

The toluene was removed from the fat by heating the extraction solution in an oil bath at 125°C. when a viscous residue was

[7] De Schweinitz, E. A., and Dorset, M., *J. Am. Chem. Soc.*, 1895, xvii, 605; 1903, xxv, 358.

[8] Final report of the Royal Commission for Tuberculosis, 1913, xl, 30.

[9] Goris, A., and Liot, A., *Ann. Inst. Pasteur*, 1920, xxxiv, 497.

obtained which partially solidified on cooling. This product, representing 124.7 gm. of extracted material, dissolved completely in 1,200 cc. of chloroform. An aliquot part of the latter solution (20 cc.), when evaporated and heated at 80°C. in a vacuum, yielded 1.9853 gm. of a dark-colored viscous product resembling crude molten rubber, which gave after incineration 0.0443 gm. of ash corresponding to 2.23 per cent of the weight of material destroyed. 87.46 per cent of this ash was composed of the following inorganic oxides: P_2O_5, 54.58 per cent; CaO, 24.28 per cent; MgO, 8.70 per cent, leaving 12.54 per cent to be accounted for in the form of sodium and potassium salts, which have been shown to be normal constituents of tubercle bacilli. Earlier investigators, who have studied the fat extracts of tubercle bacilli, have paid little attention to the presence of inorganic elements with the exception of phosphorus and nitrogen.

Goris,[9] for example, extracted the bacilli with acetone and examined the ash after evaporation and incineration, but claimed that it was composed chiefly of phosphorus. The fact that calcium and magnesium together with phosphorus are carried into solution with fat in such large quantities is a fact of considerable interest. Whether the alkali earths are present here in the form of salts of organic acids, or result from incineration of nucleoprotein of tubercle bacilli soluble in toluene or carried in colloidal suspension remains to be decided by further research.

Extraction of Defatted Tubercle Bacilli with 95 Per Cent Alcohol.

To determine if alcohol-soluble material is present in tubercle bacilli after removal of fat with toluene 61 gm. of extracted Parke, Davis and Co. bacilli were digested with 300 cc. of hot 95 per cent alcohol for 3 hours. This operation was repeated three times when we obtained a deep red solution. After filtering and drying the residue at 80°C. in a partial vacuum we recovered 56 gm. of bacilli showing a loss of 8.2 per cent. This residue yielded a colorless, odorless powder when pulverized and on analysis for inorganic constituents gave the following values: N, 9.61 per cent; ash, 5.31 per cent; P_2O_5 in ash, 41.7 per cent, CaO in ash, 1.6 per cent; MgO in ash, 7.57 per cent.

In other words, inorganic material is dissolved by extraction with alcohol and the amount of P_2O_5 extracted equals about 83.75

per cent of the weight of ash removed or 5.8 per cent of the total loss in weight. This large loss in phosphorus by solution in alcohol indicates removal from the tubercle bacilli of products of the nature of phosphatides. This alcohol extract deposited a white flocculent precipitate on cooling, and a similar precipitation was produced by diluting the alcohol with water. It will be of interest to learn whether alcohol-soluble proteins are extracted from tubercle bacilli by this treatment and just as soon as sufficient tubercle bacilli are available for research we shall investigate this problem.

Separation of Tuberculinic Acid.

The material used for the preparation of nucleic acid (tuberculinic acid) was the dried tubercle bacilli left after extraction with toluene as described above. The hydrolysis of the nucleoprotein was effected by treatment of the dried bacilli with 3 per cent aqueous sodium hydroxide for 1 hour. Several preliminary experiments showed that one treatment with this alkali solution was not sufficient to remove all the nucleic acid, and we found it advisable and practical to apply a second alkali treatment with 5 per cent sodium hydroxide solution. Our tests indicated that this removed completely the nucleic acid. Lack of time and the small amount of tubercle bacilli available prevented us from studying the conditions influencing hydrolysis as carefully as we desired, but from the results which we were fortunate in obtaining it can be said that the yield of nucleic acid obtained by us was about 1 per cent of the weight of the original tubercle bacilli. This probably does not represent the total amount of nucleic acid present, and if more is contained in the bacilli it is either destroyed by the treatment with alkali, or remains in the filtrate after precipitation with alcohol. In support of this conclusion is our observation that the residue left after alkali hydrolysis gave none of the specific tests for pyrimidines. The nucleic acid (tuberculinic acid) was obtained in the form of a light grey powder. It was not subjected to further purification before a hydrolysis for the separation and identification of pyrimidines. Just as soon as it is possible to obtain more of this acid it will be carefully purified and submitted to a complete analysis.

Our method of operating for the preparation of this nucleic acid is revealed by the description of a single hydrolysis experiment:

100 gm. of dry, defatted tubercle bacilli are thoroughly triturated in a large mortar with 1,600 cc. of water. A solution of sodium hydroxide containing 60 gm. of NaOH in 400 cc. of water is then added and the bacilli are thoroughly ground in suspension for 1 hour. This grinding is absolutely essential in order to break up the cells and get good penetration of the alkali into the cellular structure. After 1 hour the solution is made distinctly acid with acetic acid, care being taken to stir continuously and vigorously during addition; at the same time keeping the temperature of the solution below 20°C. After acidifying, the mixture is then allowed to stand, generally over night, and finally filtered and the residue washed carefully with water containing about 2 per cent of acetic acid. This acid filtrate is saved, as described below.

The residue of bacilli is given a second treatment with 5 per cent sodium hydroxide solution using exactly 1,000 cc. and applying the trituration treatment at ordinary temperature for 2 hours. Acetic acid is then added as before and the filtrate saved. From the 100 gm. of dried bacilli we recovered 84 gm. of insoluble material, indicating that 16 per cent of the original bacilli was extracted by the alkali. This residue contained 1.19 per cent of P_2O_5 while the original nucleoprotein used for hydrolysis contained 2.19 per cent of P_2O_5. Therefore, only 45.66 per cent of the phosphorus was removed by alkali. Since this residue failed in every one of our hydrolysis experiments to give positive tests for pyrimidines, we concluded that the extraction of nucleic acid was complete, and that the phosphorus present in the residue (54.34 per cent) is held in the form of other combinations beside nucleic acid.

Precipitation of Tuberculinic Acid.

The acetic acid filtrate obtained from the hydrolysis of the nucleoprotein with 3 per cent alkali is diluted with concentrated hydrochloric acid until a slight permanent precipitate is produced, when an equal volume of 95 per cent alcohol is then stirred into the solution. Care should be taken to keep the mixture cold during this operation. The solution is then allowed to stand for several hours when the nucleic acid deposits as a layer or amor-

phous precipitate on the bottom of the precipitating jar. The clear supernatant liquid is easily removed by decantation and the nucleic acid then separated by centrifuging. This is triturated with 50 per cent alcohol and the separation made again by means of the centrifuge. After repeating this operation three times using 80 per cent, 95 per cent, and anhydrous alcohol, respectively, a product was obtained which was considered of sufficient purity for hydrolysis. The acid was washed with ether and finally dried in a vacuum over concentrated sulfuric acid. The filtrate obtained after the second alkali treatment with 5 per cent alkali was worked up in a similar manner for nucleic acid, but the yield from this fraction was very small in every case. By application of the above procedure to 436 gm. of fat-free and dried tubercle bacilli we succeeded in isolating 7.7 gm. of *tuberculinic acid*. This product failed to respond to the biuret test for proteins and gave all the reactions and tests which are characteristic of nucleic acids.

Nitrogen Distribution in the Protein of Tubercle Bacilli after Removal of Nucleic Acid.

Analysis was made according to the method of Van Slyke.

	gm.	per cent
Total nitrogen	0.56354	100.00
Amide nitrogen	0.06665	11.83
Humin "	0.02316	4.11
Cystine "	0.00710	1.26
Arginine "	0.05990	10.63
Histidine "	0.06470	11.48
Lysine "	0.02080	3.69
Monoamino nitrogen	0.26710	47.39
Non-amino "	0.05265	9.34
Tryptophane	Present.	
Total nitrogen accounted for	0.56206	99.73

SUMMARY.

1. Tubercle bacilli (human and bovine) have been carefully extracted with toluene to remove fat and the cellular structure subjected to hydrolysis at ordinary temperature with 3 per cent sodium hydroxide to separate the nucleic acid.

2. The nucleic acid of tubercle bacilli has been precipitated by means of alcohol and 7.7 gm. obtained which failed to respond to the biuret test for proteins.

3. This specific nucleic acid has been given the name, *tuberculinic acid*.

4. The protein residue left behind after removal of tuberculinic acid has been analyzed according to the method of Van Slyke and its total nitrogen accounted for.

THE PYRIMIDINES CONTAINED IN TUBERCULINIC ACID.
THE NUCLEIC ACID OF TUBERCLE BACILLI.*

By TREAT B. JOHNSON and ELMER B. BROWN.

(*From the Department of Chemistry, Yale University, New Haven.*)

A careful review of the literature of tuberculosis and especially that dealing with the chemistry of tubercle bacilli has revealed the fact that we have not at present an accurate knowledge of the constitution of the pyrimidines which function in the molecular structure of the nucleic acid of tubercle bacilli. Our knowledge of the nature of the sugar content of this organism is likewise equally scanty, while in the case of the purines it has been definitely established that the normal constituents of nucleic acids, guanine and adenine, are present.

Levene,[1] while working with defatted tubercle bacilli, attempted to isolate the pyrimidines contained in that organism, but the products which he obtained after hydrolysis were so impure that it was impossible for him to decide conclusively as to their exact constitution. He separated from his pyrimidine fractions a nitrogenous substance which gave analytical values for nitrogen intermediate between the theoretical values for thymine and uracil, and he concluded that he was dealing with a mixture of these two compounds. Levene also examined his solution for bases, but was unable to draw any definite conclusion as to the occurrence of cytosine. This investigator, as well as all others who have worked in this field, was greatly handicapped in his research by not having at his service the characteristic color tests, which have since been developed for the detection and identification of uracil I, thymine II, and cytosine III.

---

* This paper is constructed from a dissertation which was presented by Elmer B. Brown in June, 1922, to the Faculty of the Yale Graduate School in candidacy for the degree of Doctor of Philosophy.

[1] Levene, P. A., *J. Med. Research*, 1904, xii, 251.

$$
\begin{array}{ccc}
\text{NH——CO} & \text{NH——CO} & \text{N}===\text{CNH}_2 \\
| \qquad | & | \qquad | & | \qquad | \\
\text{CO} \quad \text{CH} & \text{CO} \quad \text{CCH}_3 & \text{CO} \quad \text{CH} \\
| \qquad \| & | \qquad \| & | \qquad \| \\
\text{NH——CH} & \text{NH——CH} & \text{NH——CH} \\
\text{I} & \text{II} & \text{III}
\end{array}
$$

Other investigators have reported the presence of thymine among the decomposition products of tubercle bacilli, but a careful review of the reports of their researches will reveal the fact that their conclusions are not supported by reliable experimental data. For example, Kitajima,[2] who separated the nucleic acid called "Tuberkelothymine Säure," states that it contained the pyrimidine, thymine, or a similar substance, but since uracil possesses many properties in common with thymine it cannot be concluded from his experimental evidence which one of these two pyrimidines he was actually dealing with.

In our preceding paper,[3] we have given a description of our method of separating tuberculinic acid from tubercle bacilli, and by the application of which we succeeded in obtaining from a mixture of human and bovine tubercle bacilli 7.7 gm. of this acid for further investigation. 0.7 gm. of this product was utilized in making certain tests to assure ourselves that we actually had in hand a nucleic acid. The remainder (7 gm.) was subjected to hydrolysis and a complete analysis made for pyrimidines. We now find, after a careful examination of the products of hydrolysis, that this nucleic acid (tuberculinic acid) is characterized by the fact that it contains no *uracil*. The only pyrimidines in the acid, for which we were able to obtain positive tests and succeeded in isolating, were *thymine* and *cytosine*. Both of these two pyrimidines were obtained in sufficient quantities for complete purification and analysis. From the new data which we have succeeded in obtaining we may conclude, therefore, that *tuberculinic acid is a nucleic acid of animal origin*, thereby establishing the natural group to which the unicellular organism *tubercle bacillus* is to be assigned.

In the experimental part of this paper is given a complete description of our method of separating the pyrimidines, *thymine* and *cytosine*, from tuberculinic acid, and of establishing their

[2] Kitajima, T., *Mitt. Med. Ges. Tokio*, 1902, xvi, 17.

[3] Johnson, T. B., and Brown, E. B., *J. Biol. Chem.*, 1922, liv, 721.

identity by characteristic color tests. The study of tuberculinic acid will be continued in the Sterling Chemistry Laboratory.

EXPERIMENTAL PART.

Hydrolysis of Tuberculinic Acid with Sulfuric Acid.

7 gm. of tuberculinic acid from tubercle bacilli were dissolved in a mixture of 42 cc. of water and 14 cc. of concentrated sulfuric acid. The solution was then heated in an oil bath at 125°C. for 25 hours. This operation was conducted in a round bottom Pyrex flask which was connected to a reflux condenser. After the hydrolysis was completed (25 hours) 500 cc. of water were added and also finely pulverized barium hydroxide until the resulting solution became distinctly alkaline towards litmus. The solution was then filtered and the precipitate washed thoroughly with hot water. The combined filtrates and washings were then concentrated under diminished pressure to a volume of exactly 250 cc., and this finally made distinctly acid with sulfuric acid and then filtered to remove barium sulfate and probably some guanine sulfate.

A saturated solution of silver sulfate (5 gm.) was then added to this sulfuric acid solution, when a voluminous, chocolate-colored precipitate was obtained. This was filtered off and finally thrown away after it was shown by tests that it contained no pyrimidines. To the clear filtrate barium hydroxide was added until the solution remained distinctly alkaline, when a voluminous white precipitate of silver salts was obtained. After thorough cooling of the solution this salt was separated with the centrifuge, washed again with water, and finally separated again with the centrifuge.

This silver salt was then triturated with 400 cc. of water, acidified with 5 cc. of concentrated sulfuric acid, and the silver removed as sulfide by thorough saturation with hydrogen sulfide. This operation required that the mixture be stirred vigorously during the treatment with hydrogen sulfide in order to break up all lumps of silver salt suspended in the solution. The silver sulfide was removed by filtration and the precipitate given a second treatment in dilute sulfuric acid solution with hydrogen sulfide at 40°, when the decomposition of silver salts of pyrimidines was considered complete. The combined filtrates from this process were then

carefully freed from sulfuric acid and barium and finally concentrated on a steam bath to a volume of 300 cc. This solution was practically colorless, and when Wheeler and Johnson's[4] color test for uracil and cytosine was applied to an aliquot part of 5 cc. a strong positive test for these pyrimidines was obtained.

Precipitation of Cytosine as Phosphotungstate.

The above solution containing pyrimidines was first concentrated to a volume of 150 cc. and then acidified with 1 cc. of concentrated sulfuric acid. Phosphotungstic acid solution was then added drop by drop as long as an immediate precipitate was produced. We were very careful to avoid adding a large excess of this reagent. A copious white precipitate was produced, and after filtering, it was washed with a small volume of cold water. The filtrate which contained thymine was saved, as described below.

The cytosine was liberated from the phosphotungstic acid precipitate by suspending the latter in 400 cc. of water and finally decomposing it with an excess of barium hydroxide. This operation is facilitated by stirring and this was continued for about 3 hours to break up thoroughly all lumps of salt. The precipitate was then filtered off and the excess of barium removed by saturating the solution with carbon dioxide. When the clear filtrate was concentrated to a very small volume and cooled, *cytosine* separated in the form of large plates. The pyrimidine was filtered off, washed with alcohol and ether, and finally dried in a vacuum over sulfuric acid. The yield was 0.1035 gm. This base gave the characteristic color test of Wheeler and Johnson,[4] and an analysis for water was in agreement with the required value for this base.

Water determination. 0.1033 gm. of base lost at 110°C. 0.0147 gm. of water.

$$C_4H_5ON_3. \quad \text{Calculated.} \quad H_2O \ 14.07.$$
$$\text{Found.} \qquad " \quad 14.23.$$

Since this is the first cytosine to be separated from the nucleic acid of tubercle bacilli the specimen has been preserved for future reference.

4 Wheeler, H. L., and Johnson, T. B., *J. Biol. Chem.*, 1907, iii, 183.

Separation and Identification of Thymine.

The filtrate left after precipitation of cytosine with phosphotungstic acid was freed from this reagent by precipitation with barium hydroxide, the excess of barium removed in the usual manner by saturating the fluid with carbon dioxide and the solution then concentrated to a volume of 5 to 10 cc. and allowed to cool. Thymine separated in the form of clusters of foliated crystals, possessing a mother of pearl luster. They were separated, washed with alcohol and ether, and then dried in a vacuum over concentrated sulfuric acid. The yield was 0.0756 gm.

Nitrogen determination (Kjeldahl).

$$C_5H_6O_2N_2. \quad \text{Calculated.} \quad N\ 22.22.$$
$$\text{Found.} \qquad \text{``}\ 22.12.$$

A portion of this thymine and an aliquot part of the filtrate from which it crystallized were tested for uracil and cytosine by application of Wheeler and Johnson's bromine reaction.[4] In neither case did we obtain the characteristic color test for these pyrimidines, thus proving that neither of them were present here as impurities.

Identification of Thymine by Means of Johnson and Baudisch's Color Reaction.

Johnson and Baudisch[5] have recently shown that the thymine molecule can be destroyed by oxidation with air in the presence of ferrous sulfate and acid sodium carbonate with cleavage of the ring and formation of pyruvic acid, acetole, urea, and formic acid. The urea is easily identified by precipitation with xanthydrol[6] and the acetole is detected by the characteristic color reactions which are developed when this compound is allowed to interact in alkali solution with o-aminobenzaldehyde. This leads to the formation of 3-oxyquinaldine which is characterized by its brilliant blue fluorescence in alkaline solution. These reactions are extremely sensitive and serve for the detection of thymine when present in very minute quantities. For example, 0.004

[5] Johnson, T. B., and Baudisch, O., *J. Am. Chem. Soc.*, 1921, xliii, 2670.
[6] Fosse, R., *Compt. rend. Acad.*, 1907, cxlv, 813; *Ann. Inst. Pasteur*, 1916, xxx, 525, 673.

gm. of our thymine obtained from tubercle bacilli was oxidized according to the directions of Johnson and Baudisch,[5] and the distillate tested according to the procedure outlined in their original paper. The characteristic blue fluorescence of 3-oxyquinaldine was easily recognized, and was greatly intensified when viewed by the light of an iron arc. The residue left after distilling to remove acetole was dissolved in 50 per cent acetic acid and the urea precipitated with xanthydrol when 0.0075 gm. of the dixanthyl-urea was obtained melting at 257°C. The result shows that 52 per cent of the total urea theoretically possible of formation by destruction of the thymine used was obtained in the form of the xanthydrol derivative.

It is a fact of considerable interest to the writers that no uracil was produced during the digestion of tuberculinic acid with sulfuric acid as a result of the hydrolysis of cytosine. Apparently this base is perfectly stable under the conditions employed in our work, and the result obtained leads the writers to believe that wrong conclusions may have been made in the past regarding the origin of this pyrimidine in several of the products examined. It would be a fact of considerable interest from a chemical standpoint if it should be established that cytosine, when linked in the nucleic acid molecule, is more resistant to the action of mineral acids than when in the free condition as a base.

Examination of the Nucleoprotein of Tubercle Bacilli for Pyrimidines after Hydrolysis and Separation of Nucleic Acid.

30 gm. of the nucleoprotein,[3] which had been analyzed according to the method of Van Slyke, were subjected to an intensive hydrolysis with 33 per cent sulfuric acid by digesting for 30 hours at 130°C. The solution was then diluted to 800 cc. with water, filtered, and the sulfuric acid removed by precipitation as barium sulfate. The excess of barium was then precipitated as carbonate and the filtrate concentrated by heating in a vacuum to a volume of 150 cc. This solution was then acidified with nitric acid and 5 gm. of silver nitrate were dissolved in it. A precipitate was formed here which was discarded, as silver salts of the pyrimidine are not precipitated in nitric acid solution. The usual procedure was then applied for precipitating the pyrimidine as silver salts in alkaline solution and the resulting silver precipitate separated

and examined for pyrimidines. It was first treated with dilute
hydrochloric acid to remove silver as silver chloride, and the fil-
trate then concentrated to a volume of 10 cc. One-half of this
solution was concentrated still further and then tested for uracil
and cytosine according to the method of Wheeler and Johnson.[4]
We obtained no indications of the formation of a purple color
proving the absence of uracil and cytosine. The remaining 5 cc.
of solution were treated according to the directions of Johnson
and Baudisch[5] to test for thymine when a negative result was
also obtained. In other words, this product (nucleoprotein)
was entirely free from nucleic acid.

SUMMARY.

1. Tuberculinic acid, the nucleic acid of tubercle bacilli, has
been submitted to acid hydrolysis by digestion with sulfuric
acid and an analysis made for pyrimidines. Cytosine and thy-
mine have been separated and identified.

2. Uracil was not found in the molecule of tuberculinic acid.

QUANTITATIVE ASPECTS OF THE RÔLE OF VITAMINE B IN NUTRITION.*

By THOMAS B. OSBORNE and LAFAYETTE B. MENDEL.

With the Cooperation of Helen C. Cannon.

(From the Laboratory of the Connecticut Agricultural Experiment Station
and the Sheffield Laboratory of Physiological Chemistry, Yale
University, New Haven.)

Students of the vitamine problem seem at present to be in accord in the belief that the now recognized food accessories are not synthesized *de novo* by the higher animals but are derived from their food supply. Furthermore, the comparatively rapid onset of symptoms of disorder following the lack of vitamine B in the diet leads to the conclusion that normal animals do not possess any large available reserve of this food factor. It is of obvious importance therefore to secure a quantitative estimate of the actual amounts of the different vitamines requisite for the proper physiologic function of the various animal organisms at all stages of their existence and under the different conditions represented by unlike age, sex, activity, diet, planes of metabolism, or other possible modifying circumstances.

So long as the vitamines remain unidentified as chemical individuals their function can only be measured in terms of the naturally occurring products or their derivatives which contain the unidentified essentials. Furthermore, it is clearly established that the vitamine potency of such products varies in different specimens of the same material and may be influenced by the mode of production and similar biological factors. These inevitable facts have made it difficult to secure satisfactory bases for comparisons involving vitamine dosage or potency.

There is no longer any question regarding the indispensability of vitamine B for the adequate nutrition of a considerable number of species of laboratory animals, particularly during the period of growth. The widely known report of the British Medical Research Committee[1] is responsible for the statement that "the requirements of the body for the factor [vitamine B] are much greater during growth than during maturity." It is not easy, however, to discover experimental evidences in proof of this thesis. Perhaps this is based on Drummond's earlier statement[2] that in the case of young rats the withdrawal of vitamine B from the dietary is almost invariably followed by an immediate cessation of growth succeeded by a period during which the animal either maintains a constant body weight or shows a very slow decline until the sudden terminal change resulting in death. Drummond adds that "from a large number of observations it has been ascertained that the period of maintenance, which follows the restriction and precedes the final decline, is roughly proportional to the age of the animal when the restriction is imposed." His further statement that "adult animals are able to live for considerable periods without receiving a supply of this dietary factor [vitamine B]" must be interpreted in the light of his added statement that "during this period they usually show a slow decline in body-weight, which is associated at a later stage with an enfeebled resistance to disease. The ultimate decline is, however, inevitable." A somewhat similar view is recorded in the British Report when it states, that "adult animals show a very gradual fall in body weight following the restriction [removal of vitamine B], but sooner or later both young and old subjects suffer a rapid decline which invariably terminates fatally." It seems scarcely justifiable, in view of the admitted fact that rats of all ages inevitably decline when vitamine B is omitted from their diet, to suggest any marked difference in the actual need of this vitamine on the basis of the differences in the *rate* of decline shown by animals possessing undoubtedly unlike nutritive reserves. It seems worth while to emphasize this criticism in view of the fact

[1] Report on the present state of knowledge concerning accessory food factors (vitamines), *Med. Research Com., Nat. Health Insurance, Special Rep. Series, No. 38*, 1919, 21.

[2] Drummond, J. C., *Biochem. J.*, 1918, xii, 29.

that published statements have awakened a widespread belief that vitamine B is essential primarily for growth.

The problem involved can be investigated experimentally. Many of the earlier studies in this field are open to criticisms in the light of the rapidly increasing knowledge shed upon this new field of research during the past few years. Consequently, it is unnecessary to present an elaborate review of the subject. Frequently the dietary materials used were not demonstrated to be as free from vitamine B as the investigations strictly required; and further, as we have often pointed out, the earlier methods of feeding have been such as to leave the actual intake of various important factors involved in the questions at issue uncertain or undetermined.

Evidence has not been lacking that the amount of growth of experimental animals on otherwise uniform and adequate diets may be proportional to the amount of vitamine B-bearing products included in the diet. For example, in 1917 we showed that in diets in which varying percentages of yeast were incorporated as the source of vitamine B in otherwise adequate food mixtures the rate of growth increased up to a certain point with the increasing content of the yeast component.[3] A recalculation of some of our earlier data so as to show the absolute daily intake of yeast in these experiments gives data which also are in harmony with the suggestion that, up to the essential minimum, growth (and consequently also food intake) improves with the increasing dosage of the source of vitamine. Similarly, regarding another source of vitamine B, McCollum[4] notes that "rats which are at all depleted with respect to the factor B do not recover when only 2 per cent of wheat germ is present in the ration. They do recover from a condition of extreme weakness, and partial paralysis on our ration with the extracted germ to the extent of but 3 per cent."

The same general parallelism of improved growth with increasing doses of vitamine B up to the essential minimum or optimum for normal response has been observed in our studies with vitamine-bearing fractions of yeast extracts, as well as in numerous investigations on the effect of varying dosage of plant products upon the

[3] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1917, xxxi, 149.

[4] McCollum, E. V., *J. Am. Med. Assn.*, 1917, lxviii, 1379.

growth of rats.[5] The facts indicated in the preceding review
could doubtless be further illustrated by reference to many
individual experiments scattered through the now voluminous
literature on vitamines.

Some time ago we undertook to ascertain the actual daily
requirement of vitamine B for rats at various stages of their
growth through the period of adolescence and in animals of widely
unlike size. The conditions selected were intended to be more
strictly comparable or identical with respect to all the other
dietary factors than had been the case in previous investigations.
These studies were expected to furnish conclusive evidence as to
whether the requirement for vitamine B actually varies with the
age (and size) of the individual; and whether there is a wide
variation in the need of this vitamine among different animals kept
under otherwise comparable hygienic and dietary conditions.   It
was anticipated, further, that such a study might throw some light
on the possible mode of action of the vitamine; thus, for example,
if it functions in somewhat the same manner as enzymes and
related catalysts are supposed to act, a definite small initial dose
might serve under a large variety of conditions to initiate a chain
of events or chemical reactions of widely different extent which
might proceed to their normal conclusion according to attendant
circumstances in the system in which they were established.   On
the other hand, it is equally conceivable that the vitamine may
act as an integral chemical component of biological mechanisms—
cells, for instance—in which the need thereof would be expressed
quantitatively by increments of demand proportional to the
increase in the structures of which they were a part—just as other
"building stones" in the organism which are indispensable to liv-
ing cells must be supplied in amounts proportional to the number
of cells creating the structural demand.   Or again, even a mere
stimulant like a drug might be required in doses proportional to
the tissue to be stimulated, analogously to what one finds in the
domain of pharmacology. Thus a dose of pilocarpin which will
promote secretion in a small animal no longer suffices in a much
larger individual. This well known fact is taken into account in

[5] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1919, xxxix, 29;
1920, xli, 451; 1920, xlii, 465; *J. Am. Med. Assn.*, 1922, lxxviii, 1121.

adjusting the dosage of medicinal agents for persons of widely unlike age and size.

<center>EXPERIMENTAL.</center>

In the present feeding experiments on white rats a *food mixture* consisting of casein 18, starch 54, lard 15, butter fat 9, and salt mixture[6] 4 per cent, and demonstrated as the result of many trials to promote growth to full adult size when an abundance of vitamine B in the form of yeast is supplied, represented the "standard food" which was always furnished *ad libitum* along with tap water to the animals. *The vitamine B product* consisted, unless otherwise specified, of tablets from a single lot of dried brewery yeast which has been used in our laboratory for several years. The individual tablets, which were manufactured in our laboratory showed a variation of not more than $\pm$ 5 per cent from the dosage size indicated for them. The yeast was fed in a separate dish and almost invariably eaten promptly as soon as it was offered.

The plan of the feeding trials consisted in continuing the same dosage of yeast daily over a period of 1 year, the beginning of such a dietary régime being made at different ages (and consequently different sizes), with animals previously maintained on the mixed diet customarily furnished after weaning to our colony.[7] In this way it became possible to ascertain not only what doses of vitamine-bearing supplement were required at different ages until adult size was reached, but also whether a more or less prolonged preliminary period of less "artificial" feeding on food mixtures which have been demonstrated to be adequate in every respect for the nutritive well being of the rat would alter the subsequent vitamine requirement. Not less than five rats (usually males) were employed in each test; and they were kept in individual cages. At the end of the year on unchanged diet (or earlier if serious decline in weight had intervened) the dosage of yeast was increased in a considerable number of cases in order to ascertain whether still greater increments of weight could be secured on the standard diet employed. The results are summarized in graphic form in Charts 1 and 2 which show the changes in body weight

[6] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1919, xxxvii, 572.

[7] Ferry, E. L., *J. Lab. and Clin. Med.*, 1919–20, v, 735.

CHART 1.

744

CHART 2.

CHARTS 1 and 2. Representing the comparative effect produced by various daily doses of dried brewery yeast, offered apart from the rest of the ration as the sole source of vitamine B, on rats receiving the otherwise uniform food mixture described in the text. Each series represents, where possible, the results of a year's feeding period beginning with animals at different sizes (40, 70, 150, and 240 gm., respectively) in the different groups. It should be noted that with the continuance of growth the dose per unit of size decreases.

745

during the year of the fixed diet. Attention is directed to the fact that the feeding trials of successive groups on the same yeast dosage were begun when the animals had reached approximately 40, 70, 150, and 240 gm., respectively, of body weight.

Limitations of space do not permit the recording here of the enormous number of data furnished by these laborious tests. In examining the charts it should be borne in mind that the average adult size for male rats in our colony approximates 300 to 350 gm.; adult size beyond which little or at best slow increments of weight occur being reached at the age of 240 to 300 days.

With these facts in mind it will be noted that in Groups 1, 2, 3, and 4, of Series A, in which 200 mg. of dry yeast were supplied daily to each rat, the animals with few exceptions reached the average adult size within the average normal time. The occasional death of a rat among the large number investigated need not militate against the significance of the general outcome of the experiments. The apparent tendency, in all of the series, for the rats started later on the "artificial" diet to grow somewhat larger than those which began to receive the standard food plus yeast at an early age, presumably is due to the differences in age at the end of the various feeding periods in the groups. Our stock rats tend to show a slow but steady increment of weight even long after the end of the first year of life. Whether this age factor is sufficient to account for this phenomenon we are unable to state at the present time. It will be noted also that the growth of an occasional animal such as one in Series A, Group 3, and one in Series B, Group 3, tended to exceed that of its mates, suggesting either unusually low vitamine requirement or exceptional capacity for growth in individual animals. On the whole, however, the results are remarkably concordant when the severe conditions of the experiment—long periods of confinement in small cages without exercise, and without change of the "purified" diet—and also the fact that the rate of growth and ultimate maximum size of our stock animals varies quite as widely, are borne in mind.

It will be seen at once that, although the daily dosage of 200 mg. of our yeast seems to suffice as a source of vitamine B to facilitate average growth at all periods until adult size is reached, the outcome with 100 mg. (Series B), particularly after the animals attain a weight of 200 gm., tends to be less satisfactory. When the

daily dosage is still smaller (50 mg. in Series C; 25 mg. in Series D), the rate of growth is more or less retarded from the very beginning of the experiments. In the case of the *larger* animals in Series D 25 mg. of yeast per day failed in every instance to secure even maintenance of body weight for any length of time, although all of the animals recovered their weight and began to grow when the vitamine B intake was augmented. Even with somewhat smaller animals on the lowest dosage not only was no long continued growth secured but maintenance became somewhat difficult.

Obviously, when the daily intake of yeast remains unchanged while an animal is rapidly altering its body weight the dosage of vitamine estimated on the basis of any acceptable "unit," whether it be in terms of weight, stature, or surface area, will become progressively altered. It is instructive, therefore, to attempt an estimate of the actual dosage in our numerous experiments under strictly comparable conditions in terms of one of these "units." For purpose of illustration we have selected 100 gm. body weight in such calculations. Estimated on this basis, which is admittedly open to theoretical objections just as are the other various units which have been proposed for the record of basal metabolism, it appears, broadly speaking, as if the vitamine B requirement in the case of the rat under conditions of growth or maintenance upon a food of constant qualitative and quantitative make-up bears some quantitative relationship to the mass of active tissue. Under the conditions of our experiment the daily requisite per 100 gm. of body weight seemed to approximate what is contained in 50 to 60 mg. of our dry yeast. This conclusion is reached by observing the weight of the animals for which maintenance is either barely possible, or no longer satisfactory, in the numerous individual experiments on the varied fixed daily doses of yeast. Table I, which receives its justification from our statistics and a study of Charts 1 and 2, summarizes some of the data applicable to the problem.

It should perhaps be specifically noted that a number of the larger animals, such as those in Series B for example, showed a satisfactory maintenance and sometimes even slow increments in weight on a daily yeast dosage which, calculated in terms of 100 gm. body weight units, was considerably less than 50 mg., the quantity which we have judged to be the approximate requirement

TABLE I.

Series	Daily dose of Yeast (mg)	Group	Original weight (gm)	At 100 gm. body weight — No. and sex of rats	Yeast per 100 gm. (mg)	Growth	At 150 gm. body weight — No. and sex of rats	Yeast per 100 gm. (mg)	Growth	At 200 gm. body weight — No. and sex of rats	Yeast per 100 gm. (mg)	Growth	At 250 gm. body weight — No. and sex of rats	Yeast per 100 gm. (mg)	Growth	At 300 gm. body weight — No. and sex of rats	Yeast per 100 gm. (mg)	Growth
A	200	1	40	6♂	200	Normal.	6♂	133	Normal.	6♂	100	Normal.	6♂	80	Normal.	5♂	66	Normal.
		2	70	6♂	200	"	6♂	133	"	6♂	100	"	4♂	80	"	4♂	66	"
		3	150							6♂	100	"	6♂	80	"	6♂	66	"
		4	240													6♂	66	"
B	100	1	40	6♂	100	1 normal. 4 nearly normal. 1 very slow.	5♂	65	1 normal. 4 nearly normal.	5♂	50	4 normal. 1 nearly normal.	5♂	40	2 normal. 3 very slow.	3♂	33	Normal.
		2	70	6♂	100	Normal.	6♂	65	Normal.	6♂	50	Normal.	5♂	40	3 normal. 2 very slow.	4♂	33	Normal.
		3	150							6♂	50	Normal.	6♂	40	Normal.	4♂	33	"
		4	240															
C	50	1	40	3♂ 3♀	50	3 nearly normal. 3 normal.	3♂ 3♀	33	1 nearly normal. 2 very slow. 3 normal.	3♂	25	1 normal. 2 very slow.	1♂	20	Very slow.			

748

2	70	5♂	50	1 normal. 4 nearly normal. 1 normal.	5♂	33	1 normal. 4 very slow. 1 normal.	5♂	25	1 normal. 4 very slow.	5♂	25	Very slow.	Very slow. Decline.
		1♀			1♀						4♂	20		
											5♂	20		
3	150		25	Very slow.	3♂	17	Very slow.	3♂	13	Decline.				
4	240	6♂						6♂	13	"				
D 25	1 40	6♂												
	3 150													
	4 240													

under the conditions noted. Similar observations have been
made in the case of a number of larger and older rats which were
made to depend upon vitamine B dosage somewhat lower than
that calculated on the 50 mg. per 100 gm. weight basis. It seems
not unlikely that this outcome is, strictly speaking, not funda-
mentally in conflict with the dosage principle enunciated; for it
may be that the larger animals are comparatively rich in fat so

COMPARATIVE EFFECTS OF DIFFERENT INTAKES OF VITAMINE B
ON RATE OF GROWTH.

CHART 3.

that the actual amount of the *active* tissues, with which the vita-
mine need is assumed to be correlated, is far smaller than the
total body weight indicates. A somewhat similar situation has
been found with respect to the so called creatinine coefficient
in man. The endogenous urinary output of this substance in
general bears a fairly constant relation to the size of the individual
as expressed by body weight; an exception has repeatedly been
found, however, in the case of corpulent individuals in whom the

coefficient is low in proportion to the estimated preponderance of
inactive adipose tissue.

One further illustration of the dependence of the rate of growth,
under otherwise comparable dietary and environmental conditions,
upon the vitamine dosage is presented in Chart 3.   This shows

the differences in the rate of growth of six animals from the same
litter, each of which was put upon our standard casein diet when
it reached a size represented by 100 gm. of body weight, the
variable being the daily dosage of yeast.   The graphic results are
self-explanatory.

The evidence for a correlation between the size of the animal
and its vitamine B requirement for securing the expected nutritive

condition (*i.e.* growth or maintenance according to the size and age of the animal) has been corroborated by feeding experiments in which more concentrated preparations of vitamine have been employed. Thus, using the yeast concentrate described by Osborne and Wakeman[8] as the vitamine B supplement to our standard food, growth usually stopped when rats reached a size somewhat less than 200 gm. in body weight on a daily dose of 40 mg. of concentrate. Growth was promptly resumed when the dose was increased. Some of these effects are illustrated in Chart 4. It is little short of surprising to see how the seemingly small variations in the intake of a product, which at best must be composed in large part of extraneous matter other than the vitamine *per se*, are attended by corresponding changes in the body weight of the animals. When the smaller doses are fed to larger animals a decline in weight ensues; with intermediate doses there may be maintenance at various levels of body weight; with the larger intake for the smaller animals growth ensues.

In the experiments here recorded the qualitative and quantitative composition of the basal food mixture has been kept unchanged. The experiments, therefore, throw no direct light upon the question as to whether the make-up of the food intake has a direct bearing upon the requirement for vitamine B. It has been claimed, for example, that by increasing the protein intake the need of the vitamine may be diminished in comparison with what pertains on diets low in protein and very rich in carbohydrate.[9] Such observations as we have made incidentally in other investigations have not given any support to this contention.

The recent studies of Cowgill[10] on dogs in the laboratory of one of us furnish added evidence, in connection with another species of animal, of a quantitative relationship between the requirement of vitamine B and the size of the animal.

[8] Osborne, T. B., and Wakeman, A. J., *J. Biol. Chem.*, 1919, xl, 383.

[9] Funk, C., and Dubin, H. E., *Science*, 1920, lii, 447.

[10] Cowgill, unpublished data.

STUDIES IN INORGANIC METABOLISM.

IV. THE INFLUENCE OF YEAST AND BUTTER FAT UPON MAGNESIUM AND PHOSPHORUS ASSIMILATION.

By L. JEAN BOGERT and RUTH K. TRAIL.

(From the Department of Food Economics and Nutrition, Kansas State Agricultural College, Manhattan.)

In recently published metabolism experiments on normal women, a noticeable lowering of the total calcium excretion and a correspondingly favorable influence upon the calcium balances were observed during the periods when either yeast or butter fat were added to a basal diet very poor in vitamines. In view of these facts, which suggest some relation between the vitamine content of the diet and the assimilation of calcium, it was considered of interest to follow the magnesium and phosphorus metabolism of these subjects during the same interval, in order to see whether the yeast or butter fat exerted any similar influence simultaneously upon the metabolism of other inorganic constituents of the diet.

The phosphorus and magnesium content of the foods (given in Table I) were determined at the time of the experiment, using the same samples of food upon which the calcium determinations were made.[1] For details as to experimental procedure see the first paper of this series[2] and the paper which contains the data concerning the influence of yeast and butter fat upon calcium assimilation.[3] For convenience in comparison, however, the final data on calcium balances are included in Tables IV and V.

[1] These analyses were done at the Agricultural Experiment Station of the Kansas State Agricultural College, according to the official methods of analysis of the Association of Agricultural Chemists. The same methods were used in the analysis of the excreta.

[2] Bogert, L. J., and McKittrick, E. J., J. Biol. Chem., 1922, liv, 363.

[3] Bogert, L. J., and Trail, R. K., J. Biol. Chem., 1922, liv, 387.

Table II shows the diet of the subjects, and also their daily intake of magnesium and phosphorus, during Periods I and III. This diet was planned so as to be very low in vitamines.   The lean beef was dissected free from fat and the vegetable fat used was pure, filtered fat separated by siphoning from commercial nut margarine, melted at 40–45°C.   Dried skimmed milk (Klim)

TABLE I.

Composition of Foods.

Food.	Magnesium.	Phosphorus.
	per cent	*per cent*
Beef...	0.028	0.214
Skimmed milk................................	0.017	0.092
Bread..	0.030	0.088
Rice...	0.035	0.091
Yeast..	0.044	0.285

TABLE II.

Basal Diets for Subjects.

Food.	Subjects I and J.*			Subjects J and K.†		
	Amount.	Mg	P	Amount.	Mg	P
	gm.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
Beef......	200	0.056	0.428	200	0.056	0.428
Skimmed milk........	250	0.042	0.230	200	0.034	0.184
Bread (white).........	250	0.075	0.220	250	0.075	0.220
Rice.................	100	0.035	0.091	100	0.035	0.091
Vegetable fat........	72			50		
Sugar...............	75			50		
Starch..............	10			10		
Daily intake......		0.208	0.969		0.200	0.923

* This diet furnished 2,391 calories, 82 gm. of protein, 92 gm. of fat, and 0.395 gm. of calcium. The yeast taken in Period II added 0.037 gm. of magnesium and 0.239 gm. of phosphorus, as well as 3.9 gm. of protein and 0.023 gm. of calcium. During Period IV, 72 gm. of butter fat were substituted for the vegetable fat in the basal diet.

† This diet furnished 2,074 calories, 81 gm. of protein, 70 gm. of fat, and 0.341 gm. of calcium. The yeast taken in Period II added 0.037 gm. of magnesium and 0.239 gm. of phosphorus, as well as 3.9 gm. of protein and 0.023 gm. of calcium. During Period IV, 50 gm. of butter fat were substituted for the vegetable fat in the basal diet.

was used and its vitamine content further reduced by cooking with rice in a pressure cooker. During Period II, six cakes of compressed yeast were consumed by each subject daily, in addition to the basal diet shown in Table II, while in Period IV, an equal weight of butter fat, similarly purified, was substituted for the vegetable fat taken during the other three periods.

TABLE III.

Magnesium and Phosphorus Excretion.

Subject.	Period.	Magnesium.					Phosphorus.				
		Urine.		Feces.		To-tal.	Urine.		Feces.		To-tal.
		gm.	per cent	gm.	per cent	gm.	gm.	per cent	gm.	per cent	gm.
I	1	0.250	27	0.669	73	0.919	1.940	59	1.332	41	3.272
	II	0.215	30	0.508	70	0.723	1.646	51	1.556	49	3.202
	III	0.202	28	0.526	72	0.728	1.884	52	1.701	48	3.585
	IV	0.234	36	0.415	64	0.649	2.035	66	1.034	34	3.069
J	I	0.241	35	0.450	65	0.691	1.945	59	1.356	41	3.301
	II	0.221	24	0.706	76	0.927	1.859	47	2.056	53	3.915
	III	0.233	33	0.477	67	0.710	2.082	61	1.339	39	3.421
	IV	0.217	35	0.398	65	0.615	2.138	66	1.093	34	3.231
K	I	0.625	60	0.419	40	1.044	2.219	71	0.875	29	3.094
	II										
	III	0.250	29	0.620	71	0.870	2.453	63	1.464	37	3.917
	IV	0.299	41	0.426	59	0.725	2.681	75	0.896	25	3.577
L	I	0.220	42	0.300	58	0.520	1.586	67	0.766	33	2.352
	II	0.194	31	0.429	69	0.623	1.905	63	1.104	37	3.009
	III	0.275	26	0.802	74	1.077	1.670	49	1.758	51	3.428
	IV	0.217	32	0.463	68	0.680	2.245	70	0.957	30	3.202

The amounts of magnesium and phosphorus excreted by the different subjects during the four 4 day periods are recorded in Table III. The urinary magnesium was very constant, in practically every instance falling between 0.050 and 0.075 gm. per day. The urinary phosphorus ranged from 0.396 to 0.670 gm. per day. The phosphorus excretion shows greater variations, not only in the actual amounts excreted in the urine but also in the distribution of the total output between the urine and the feces, than are found

in either the magnesium or calcium excretion. It should be noted, however, that the larger amounts of phosphorus metabolized render these fluctuations proportionally less important.

The average percentage of the magnesium output excreted by way of the urine was 34 per cent (range 24 to 60 per cent). It is interesting to observe that the urinary magnesium formed about the same proportion of the total output in these four subjects as did the urinary calcium,[3] which averaged 37 per cent (range 24 to 54 per cent) of the total calcium excretion. On the other hand, a much larger relative amount of the phosphorus excreted was found in the urine (average 61 per cent, range 47 to 75 per cent).

Urinary calcium was decidedly greater than urinary magnesium. The ratio tended to be fairly constant for each subject, usually falling between 1:2 and 1:3. In only one case was urinary magnesium greater than calcium (Subject K, Period I), and in this instance the ratio was practically 1:1. In every case the fecal magnesium and total magnesium excretion were much less than the corresponding figures for calcium, the ratio being quite consistently about 1:2 to 1:2.5.

There seems to have been no noticeable or consistent effect produced upon the urinary magnesium or phosphorus by the yeast added to the diet in Period II, and the urinary magnesium also seems to have been unaffected by the substitution of butter fat for vegetable fat in the diet during Period IV. The urinary phosphorus, however, was increased in every case in Period IV, when a decrease in fecal phosphorus also occurred. This increase in the percentage of the phosphorus output excreted by way of the urine might be interpreted as indicative of an improved absorption of phosphorus from the intestine during Period IV, when butter fat was taken.

Both the fecal excretion and the total excretion of magnesium and phosphorus were increased in two subjects during Period II, which may be attributed chiefly to the appreciable amounts of these elements, especially phosphorus, contained in the yeast added to the diet. However, a tendency to excrete a smaller proportion of the intake, which was noted in the case of the calcium metabolism, is again apparent, so that the net result is an improved balance in every case but one (Subject J, magnesium balance). In the third subject (Subject I), the tendency toward

lowered excretion more than compensated for the increased intake of magnesium and phosphorus during Period II, with a resulting diminution in both the fecal and the total excretion of these elements. It is regrettable that it was impossible to follow the magnesium and phosphorus metabolism of Subject K during Period II, since all of the feces from this period had been used in making the calcium determinations. However, since this subject showed the typical decrease in excretion and favorable influence upon the balances of all three of these elements in Period IV, and since the magnesium and phosphorus metabolism were closely parallel to that of the calcium throughout the other three periods, it is probable that the phosphorus and magnesium balances would have shown the same improvement exhibited by the calcium balance for this subject in Period II.

Alterations in the fecal excretion and total excretion of magnesium and phosphorus were more apparent and more uniform during Period IV. The inorganic intake was not affected by the substitution of butter fat for the vegetable fat. In all four of the subjects studied, both the magnesium and phosphorus excreted in the feces and the total output of these elements were distinctly lowered during Period IV.

The effects of the changes in the diet in Periods II and IV are best seen, however, by inspection of the magnesium and phosphorus balances. These effects are not only of sufficient magnitude and uniformity as to be unmistakable, but they are exactly parallel to the effects upon the calcium balances of these subjects observed earlier. The intake, output, and balances, for the various periods are recorded in Table IV, while the average daily balances are shown in Table V. The corresponding figures for calcium metabolism are included for comparison. During Periods II and IV, there was an improvement in the balances of both magnesium and phosphorus over those of the preceding periods in every case but one. This usually took the form of increased positive balances. The phosphorus balances were all positive except that of Subject K, Period III. The magnesium balances were negative in only four instances (Period I, Subjects I and K; Period III, Subjects K and L). Whenever negative balances occurred, the subjects were on the basal diet, and these negative balances were converted to positive ones upon the addition of

yeast or butter fat to the diet in the experimental periods. The calcium balances were all negative on the basal diet and the improvement noted upon the introduction of yeast or butter fat into the diet usually consisted in a distinct lowering of negative balances, but in three cases the influence was sufficient to throw the subject into positive calcium equilibrium. The prompt return of each of the subjects to the less favorable balances of

TABLE IV.

Calcium, Magnesium, and Phosphorus Balances by Periods.

Subject.	Period.	Calcium.			Magnesium.			Phosphorus.		
		In-take.	Out-put.	Balance.	In-take.	Out-put.	Balance.	In-take.	Out-put.	Balance.
		gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.
I	I	1.580	2.059	−0.479	0.832	0.919	−0.087	3.876	3.272	+0.604
	II	1.672	1.707	−0.035	0.980	0.723	+0.257	4.832	3.202	+1.630
	III	1.580	1.806	−0.226	0.832	0.728	+0.104	3.876	3.585	+0.291
	IV	1.580	1.584	−0.004	0.832	0.649	+0.183	3.876	3.069	+0.807
J	I	1.580	1.719	−0.139	0.832	0.691	+0.141	3.876	3.301	+0.575
	II	1.672	1.784	−0.112	0.980	0.927	+0.053	4.832	3.915	+0.917
	III	1.580	1.662	−0.082	0.832	0.710	+0.122	3.876	3.421	+0.455
	IV	1.580	1.515	+0.065	0.832	0.615	+0.217	3.876	3.231	+0.645
K	I	1.364	1.588	−0.224	0.800	1.044	−0.244	3.692	3.094	+0.598
	II	1.460	1.295	+0.165						
	III	1.364	1.822	−0.458	0.800	0.870	−0.070	3.692	3.917	−0.225
	IV	1.364	1.605	−0.241	0.800	0.725	+0.075	3.692	3.577	+0.115
L	I	1.364	1.415	−0.051	0.800	0.520	+0.280	3.692	2.352	+1.340
	II	1.460	1.383	+0.077	0.948	0.623	+0.325	4.648	3.009	+1.639
	III	1.364	1.700	−0.336	0.800	1.077	−0.277	3.692	3.428	+0.264
	IV	1.364	1.415	−0.051	0.800	0.680	+0.120	3.692	3.202	+0.490

all three elements in Period III is also noteworthy. The only instance where the balance was not favorably influenced by the addition of yeast or butter fat is in Period II, Subject J, where neither the magnesium nor calcium balances responded favorably, although the phosphorus balance was increased as in the other subjects.

The parallel course of the calcium, magnesium, and phosphorus metabolism, as well as the favorable influence exerted upon the

TABLE V.

Average Daily Balances.

Period.	Calcium.					Magnesium.					Phosphorus.				
	I	J	K	L	Average.	I	J	K	L	Average.	I	J	K	L	Average.
	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.
I	−0.129	−0.035	−0.056	−0.013	−0.056	−0.022	+0.035	−0.061	+0.070	+0.005	+0.151	+0.144	+0.149	+0.335	+0.195
II	−0.009	−0.028	+0.041	+0.020	+0.006	+0.064	+0.013		+0.081	+0.053	+0.407	+0.229		+0.409	+0.348
III	−0.056	−0.021	−0.115	−0.084	−0.069	+0.026	+0.030	−0.017	−0.069	−0.007	+0.073	+0.113	−0.056	+0.066	+0.049
IV	−0.001	+0.016	−0.060	−0.013	−0.014	+0.046	+0.051	+0.019	+0.030	+0.037	+0.202	+0.161	+0.029	+0.122	+0.128

balance of each of these elements during Periods II and IV, may be seen readily from the curves shown in Chart 1, in which the average daily balances of all three elements throughout the four periods have been plotted.   The outstanding feature of this figure is the rise of the curves for all three elements during Periods II

CHART 1.   Average daily balances of phosphorus, magnesium, and calcium of all subjects.

and IV, thus indicating increased retention or diminished excretion of each of these elements when yeast or butter fat was introduced into the diet.   The favorable influence of the yeast seems to have been somewhat greater than that of the butter fat.   The fall in the curves of all three elements in Period III is also worthy of note.

While it is most interesting to observe that the influences, which favored calcium retention upon the introduction of yeast or butter fat into the diet, exerted a similar influence upon magnesium and phosphorus metabolism, we do not feel justified in concluding that these effects are necessarily due to the vitamines added to the diet, as other factors may possibly be operative.

CONCLUSIONS.

1. The magnesium and phosphorus balances in two normal women were favorably influenced by the addition of yeast to a diet of white bread, rice, lean beef, skimmed milk powder, purified nut margarine, sugar, and starch. The calcium balances, determined over the same period on the same subjects and previously reported, were similarly affected. A third subject failed to show improvement of calcium or magnesium balances upon the addition of yeast to the diet although the retention of phosphorus was favored under these circumstances.

2. The substitution of an equal weight of purified butter fat for the vegetable fat in the basal diet led to diminished fecal and total excretion of magnesium and phosphorus on a constant intake, with a favorable influence upon the balances of these elements in all four subjects studied. In every case the calcium balances showed a simultaneous improvement, the metabolism of these three elements being closely parallel throughout the experiment.

3. Although the above facts strongly suggest some relationship between the vitamine content of the diet and the assimilation of calcium, magnesium, and phosphorus, the effects produced by the yeast and butter fat may be due to factors other than their vitamine content.

VITAMIN A CONTENT OF LARD OBTAINED FROM HOGS ON A CONTROL RATION.

By MARGUERITE G. MALLON and MARJORIE CLARK.

(*From the Laboratory of Food Chemistry, Department of Home Economics, Purdue University, Lafayette.*)

There has been much discussion both by American and English investigators concerning the vitamin A content of lard. The problems concerned have been whether or not the deficiency of this factor, if there is a deficiency, is due to the lack of its presence in the original fat or to destruction of this vitamin in the commercial process of manufacture of lard. The latter includes the problem of the effect of heat and oxidation on vitamin A.

EXPERIMENTAL WORK.

The object of the experiment was to test the activity of lard prepared from hogs whose diet was known.

Two hogs were secured from the Animal Husbandry Department of the Purdue Experiment Station whose diet was known. From July 2 to September 26, 1921 these two hogs were fed on a full allowance of shelled yellow corn and clover pasture. From September 26 to December 12, the time they were killed, they were fed a full feed of yellow corn in a dry lot. These animals were unusually healthy.

In 1920 Osborne and Mendel (1) and Steenbock and Gross (2) demonstrated the presence of vitamin A in clover. The presence of this vitamin in yellow corn has been demonstrated by Steenbock and Boutwell in 1920 (3).

The fat from these hogs was rendered in our laboratory. The lard which was used was prepared from the leaves and back fat. The fat was cut in small pieces and finely divided by putting it through a food chopper. It was then filtered through filter paper above the melting point of the lard, kept in an electric oven (4).

This was done in order to obtain all the glycerides of the hog fat, and to eliminate the factors of high temperature and stirring. This filtered fat or lard of both leaves and back fat was mixed and kept for 3 months up to and during the period of the experiment in the cold storage room of the Dairy Department.

Experimental diet.*		Control diet.*	
	per cent		*per cent*
Lard.........................	30	Lard.......................	25
Corn-starch..................	48	Butter fat..................	5
Casein......................	18	Corn-starch...............	48
Salt mixture.................	4	Casein.....................	18
		Salt mixture..............	4

* In each diet 0.6 gm. yeast was fed separately, daily.

The corn-starch used was "Argo."[1] This was boiled in a reflux condenser three successive times, 1 hour each time, with equal parts of 95 per cent alcohol[2] and commercial anhydrous ether.[3] It was then washed and dried in an electric oven at 100°C. This was done to remove any vitamin A which might be present.

The casein, "from milk, washed"[3] was prepared in the same way.

The salt mixture used was the one used by Osborne and Mendel (5).

Fleischmann's compressed yeast was used as a source of vitamin B. It was dried at 110°C. over night, pulverized, and then treated for removal of vitamin A.

The butter fat used for the control rats was prepared by the usual method.

A careful record of the gram weight of the food eaten each day by each animal was taken. On account of the pasty consistency there was no scattering. The yeast, food, and distilled water were kept in separate containers.

The initial weight for both groups averaged 50 gm. The animals were weighed twice a week and the weights recorded. Each rat was kept in a separate cage.

[1] Corn Products Company.
[2] U. S. Industrial Alcohol Company.
[3] Eimer and Amend.

Two groups of animals were fed, four in the control group and four in the experimental group. In each group were animals of both sexes. The experimental animals and the control animals were kept side by side in a well lighted and well ventilated room.

DISCUSSION.

The percentage of fat fed in the experimental diet was large, 30 per cent. The rats on the experimental diet and on the control diet ate practically the same amount of food each day for 2 months.

At the end of 2 months the experimental and control animals had attained practically the same weight, the average being 153 gm. The animals were in a healthy condition; the eyes bright and clear; and the hair was smooth and glossy.

Suddenly, 2 days after the above observation the four experimental animals on the entire lard diet began to show inflammation of the eyes which developed into xerophthalmia. There was a decline in food intake and weight after the eye disease started. Two of the experimental animals died. After the xerophthalmia had fully developed, the other two animals of the experimental group were given 0.5 gm. of butter fat daily for 3 days and the eyes cleared up and became somewhat beaded.

The control animals did not develop xerophthalmia and maintained their healthy condition. The stock animals, *i.e.* the parent animals of both the experimental and control groups, were fed a ration which contained yellow corn-meal, oil meal, crude casein, alfalfa meal, NaCl, and $CaCO_3$ (Steenbock). And from time of impregnation the mother rats were given milk *ad libitum*. The good condition of the experimental animals for 2 months seems to indicate that the animals had stored in their bodies (6) from the mother's milk a sufficient amount of vitamin A for growth for some time, but the quantitative supply was not sufficient to prevent susceptibility to xerophthalmia.

CONCLUSION.

The result of this experiment seems to indicate that lard, made from leaves and back fat of hogs whose diet contained vitamin A, does not contain an adequate supply of this vitamin to prevent xerophthalmia even when fed in large amounts.

BIBLIOGRAPHY.

1. Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1920, xli, 549.
2. Steenbock, H., and Gross, E. G., *J. Biol. Chem.*, 1920, xli, 149.
3. Steenbock, H., and Boutwell, P. W., *J. Biol. Chem.*, 1920, xli, 81.
4. Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1915, xx, 379.
5. Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1919, xxxvii, 557.
6. Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1920–21, xlv, 277.

A COMPARISON OF THE DU BOIS AND THE HARRIS AND BENEDICT NORMAL STANDARDS FOR THE ESTIMATION OF THE BASAL METABOLIC RATE.

By WALTER M. BOOTHBY and IRENE SANDIFORD.

(From the Section on Clinical Metabolism, Division of Medicine, Mayo Clinic and Mayo Foundation, University of Minnesota, Rochester.)

One of the most important contributions to the science of medicine within recent years was the establishment by Lusk and Du Bois and their associates of a normal standard of heat production of sufficient accuracy to allow the general introduction of indirect calorimetry into clinical medicine.[1] The historical details, which need not be repeated here, of the development of Rubner's law of body surface into an accurate standard for the determination of the basal metabolic rate have been extensively covered in the various articles of Lusk and Du Bois and of Harris and Benedict; the former emphasize its consistencies and general applicability and the latter emphasize the point "that the metabolism or heat output of the human body even at rest does not depend on Newton's law of cooling and, therefore, is not proportional to the body surface." Murlin has recently demonstrated some of the fallacies in the position maintained by Benedict and his associates both by mathematical and by teleological arguments.

Because of their reluctance to accept the body surface law as a basis for the establishment of normal standards for heat production Harris and Benedict, in an exhaustive mathematical consideration of the subject, developed a series of biometric correlation formulas, based on stature, body weight, sex, and age (the same factors as used by Du Bois) by which "results as good as, or better than, those obtainable from the constant of basal metab-

[1] For bibliography on this subject see the following paper (Boothby, W. M., and Sandiford, I., *J. Biol. Chem.*, 1922, liv, 783).

olism per square meter of body surface can be obtained by bio-
metric formulas involving no assumption concerning derivation
of surface area but based on direct physical measurements."
Shortly after the appearance of the Harris and Benedict standards
we tabulated 404 determinations of the basal metabolic rate
expressed in percentages above and below normal, using both the
standards of Du Bois and of Harris and Benedict. The average
rates of all the cases show that those obtained by the Harris and
Benedict method are 6.5 points higher than the rates obtained
by the Du Bois method. The striking parallelism between the
results obtained by the two methods is shown in Table I in which
it is seen that 195 of the 404 determinations are within ± 2.5 of

TABLE I.

*Comparison of Metabolic Rates Obtained by the Harris and Benedict
Method with Those Obtained by the Du Bois Method.*

Difference between the Harris and Benedict rates from Du Bois' rates as standard.	No. of determinations for each range.
−10 to − 6	3
− 5 to − 1	20
0 to + 3	88
+ 4 to + 9	195*
+10 to +14	69
+15 to +19	24
+20 to +25	5

Average of 404 determinations +6.5

* Within ± 2.5 of the average deviation.

the average variation. Because of the demonstrated agreement
between the two methods in such a large proportion of subjects
we concluded that there was a fundamental similarity between
the two methods, but reserved our final opinion until after an
analysis of the discrepancies.

Means and Woodwell have recently made a similar, but some-
what more extensive analysis and have likewise shown that the
Harris and Benedict standards give basal metabolic rates on an
average 6 per cent higher than the Du Bois standards. They
point out that these differences might be abolished by an empirical
reduction in the Du Bois standards, but refrain from doing so and
recommend that the use of the Du Bois method be continued,
unchanged.

Unfortunately, and without the authorization or knowledge of Du Bois, Sanborn, in his compilation of "basal metabolism" has presented a table containing the Du Bois normal standards with 1.8 calories arbitrarily deducted. Although it is possible that in the future the present Du Bois standards may be modified, yet in order to avoid confusion it is highly desirable that they be altered as infrequently as possible.

As we have pointed out an analysis of the Du Bois and of the Harris and Benedict standards by Boothby and Sandiford and by Means and Woodwell showed for the majority of determinations a remarkable parallelism between the two methods; however, in our series materially discordant results were obtained in approximately one-fifth of the subjects. The present paper is a more extended analysis of the two standards with an attempt to learn the cause of these larger discrepancies.

Harris and Benedict claim that their standards are in no way based on Rubner's law of surface area. While this is true as far as the original construction of their formulas is concerned, yet the startling fact remains, as the curves in Chart 1 of Du Bois' paper on the basal metabolism in fever indicate, that the surface area can be calculated within certain range by their formulas with practically the same accuracy as by the formulas of Du Bois and Du Bois. The basal heat production for males as predicted by Harris and Benedict is

$$h = 66.4730 + 13.7516w + 5.0033s - 6.7550a \tag{1}$$

where h = basal heat production for 24 hours, w = weight in kilos, s = height in centimeters, and a = age in years. This formula can be transposed into one which will predict surface area by substituting constants as follows:

$$S.A. = \frac{66.4730 + 13.7516w + 5.0033s - K_1}{K_2} \tag{2}$$

where $K_1 = 141.86$ which is 6.7550 (the Harris and Benedict age factor for males) $\times$ 21 years and $K_2 = 948$ which is Du Bois' normal calories per hour for each square meter for males 21 years old multiplied by 24 hours (39.5 $\times$ 24), and $S.A.$ = surface area in square meters. This formula can be simplified by factoring and dropping insignificant figures to

$$S.A. = \frac{2.75w + s - 15.1}{190} \tag{3}$$

CHART 1. Surface areas for increasing weights at constant heights. Curves A, B, and D represent the surface area at a constant height of 151 cm. plotted for increasing weights; Curves C, E, and F are similar for a constant height of 200 cm. Curves A and E are the surface areas calculated by the Du Bois height-weight formula and are the same for men and women ($S. A. = 0.007184 \times w^{0.425} \times s^{0.725}$). Curves D and F are the surface areas calculated by the formula $\left(S.A. = \dfrac{2.75\,w + s - 15.1}{190} \right)$ derived from the Harris and Benedict heat prediction formula for men. Curves B and C are the surface areas calculated by the formula $\left(S.A. = \dfrac{5.17\,w + s + 301}{480} \right)$, derived from the Harris and Benedict heat prediction formula for women. Similar curves plotted for intermediate heights are not indicated because it is evident that they would show correspondingly smaller variations in the surface area as calculated by the three formulas.

Similarly for females:

$$h = 655.0955 + 9.5634w + 1.8496s - 4.6756a \qquad (4)$$

and by substitution of constants

$$S.A. = \frac{655.0955 + 9.5634w + 1.8496s - K_3}{K_4} \qquad (5)$$

where $K_3 = 98.19$ which is 4.6756 (the Harris and Benedict age factor for females) $\times$ 21 years and $K_4 = 888$ which is 37.0 calories per square meter (the Du Bois standard for females 21 years old) $\times$ 24 hours. By factoring and dropping insignificant figures this formula may be simplified to:

$$S.A. = \frac{5.17w + s + 301}{480} \qquad (6)$$

Formulas 3 and 6 are comparable to the height-weight formula for surface area of Du Bois and Du Bois.

$$S.A. = 0.007184 \times w^{0.425} \times s^{0.725} \qquad (7)$$

In order to test the accuracy of these formulas we have utilized the group of subjects studied by Benedict in the development of his photographic method and also the group whose surface areas were accurately measured by means of molds by Du Bois. In Table II are assembled the surface areas for these subjects calculated by (1) the formulas derived from the Harris and Benedict formulas for the prediction of total calories in men and women (Formulas 3 and 6); (2) the Du Bois linear formula; (3) the Du Bois height-weight formula; (4) Benedict's photographic method; and (5) the actual measurement by molds. An examination of Table II shows such slight differences in normal adults between the surface areas as calculated by the various methods that it is doubtful as to which formula is the most accurate. The practical conclusion which is revealed in Table II is that the surface area formulas derived from the Harris and Benedict correlation formulas for the prediction of basal heat production are possibly nearly as good for the estimation of the surface area within the average ranges of adult height and weight as is the Du Bois height-weight formula. The surface area for infants, however, cannot be predicted by the derived Harris and Benedict formula for females, as illustrated by Anna M.; also it cannot be extended to short subjects like Benny L. There also is a considerable discrepancy in Subject 19 who is likewise rather short.

TABLE II.

Comparison of the Surface Areas Calculated by Various Methods.

1	2		3	4	Harris-Benedict		7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
Subject.	Age.	Sex.	Weight.	Height.	Formula 3 for males.	Formula 6 for females.	Du Bois linear.	Du Bois height-weight.	Photographic.	Measured.	$\frac{5-7}{7}$	$\frac{5-8}{8}$	$\frac{5-9}{9}$	$\frac{9-7}{7}$	$\frac{8-9}{9}$	$\frac{8-7}{7}$	$\frac{6-9}{9}$	$\frac{6-7}{7}$	$\frac{6-8}{8}$	$\frac{5-10}{10}$	$\frac{6-10}{10}$	$\frac{7-10}{10}$	$\frac{8-10}{10}$
No.	yrs.		kg.	cm.	sq. m.	sq. m.	sq. m.	sq. m.	sq. m.	sq. m.	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent
Males.																							
1	30		111.6	166.6	2.89	2.53	2.69	2.40	2.69		+7	+20	+7	0	−11	−11	−6	−6	+5				
2	45		87.1	182.2	2.11	1.94	2.10	2.08	2.12		+2	+3	+1	+1	−2	−1	−8	−8	−7				
3	39		84.8	166.3	2.03	1.89	2.04	1.92	2.00		−1	+6	+2	+2	−4	−6	−6	−7	−2				
4	23		70.4	170.8	1.85	1.74	1.91	1.81	1.91		−3	+2	−3	0	−5	−5	−9	−9	−4				
5	21		69.0	185.4	1.90	1.76	1.95	1.91	1.93		−3	−1	−2	−1	−1	−2	−9	−10	−8				
6	48		60.4	179.7	1.75	1.65	1.74	1.75	1.72		+1	0	+2	−1	+2	+1	−4	−5	−6				
7	23		59.8	172.9	1.70	1.63	1.74	1.70	1.80		−1	0	+6	+3	−6	−2	−9	−6	−4				
8	36		59.1	176.1	1.71	1.63	1.71	1.72	1.70		0	−1	+1	−1	−1	+1	−4	−5	−5				
9	27		57.5	173.5	1.67	1.61	1.73	1.68	1.71		−1	−1	−2	−1	+1	+3	−6	−7	−4				
10	26		57.1	174.3	1.67	1.60	1.67	1.68	1.65		0	−1	0	0	−1	−1	−4	−4	−4				
11	24		53.6	167.5	1.59	1.55	1.59	1.59	1.61		−7	0	−4	+4	+4	0	−6	−3	−3				
12	55		49.5	166.4	1.52	1.51	1.63	1.53	1.61		+1	−1	−6	−1	−5	−6	−6	−7	−1				
13	37		48.0	164.8	1.49	1.49	1.47	1.50	1.54		−1	−1	−3	+5	−3	+2	−3	+1	−1				
14	24		32.9	148.1	1.18	1.29	1.19	1.19	1.20		−1	−1	−2	+1	−1	0	+8	+8	+8				

772

Females.	15	50		99.1	144.7	1.72	2.12	2.00	2.09	1.86	2.11		+1	+14	+1	+1	−12	−11	−5	−4	+8	0	0	−1
	16	10.5		86.5	144.3	1.93	1.86	2.00	1.75	1.92			−4	+10	+1	−4	−9	−13	−3	−7	+6	+3	+1	+1
	17	24		56.6	159.6	1.59	1.57	1.65	1.57	1.61			−4	+1	−1	−2	−3	−5	−3	−5	0	−3	−4	−3
	18	56		49.1	156.6	1.46	1.48	1.53	1.46	1.50			−5	0	−3	−1	−3	−5	−1	−3	+1	+2	−1	+1
	19	6.5		44.3	126.8	1.23	1.37	1.29	1.21	1.30			−5	+2	−5	+1	−7	−6	+5	+6	+13	−1	0	+1
	20	35		37.1	151.7	1.26	1.34	1.31	1.27	1.32			−4	−1	−5	+1	−4	−3	+2	+2	+6	+1	0	+1
Average deviation for males and females													2.8	3.2	2.9	1.6	1.3	4.25.4	5.7	1.8	1.8	0	+4	−1
" " "													3.0	1.7	2.1	1.5	3.4	2.96.3	6.1	1.1	1.1	+3	−4	+1
" " females													3.5	4.2	2.7	1.8	6.3	2.23.2	4.5	5.7	5.7	−3	+8	−3
Benny L.	21	56	M.	24.2	110.3	0.85	120.8	50.84	0.85		0	+1	(+32)*	(+33)*	0	(+32)*	−1	(+32)*	+2.2
Morris S.	22	21	"	61.0	161.3	1.72	66.1	69	1.69	1.67	+2	+2	−1	−2	+3	−2	−1	+1	3.1
R.H.H.	23	32	"	64.1	178.0	1.79	69.1	77	1.79	1.84	+1	0	−5	−6	−3	−5	−4	−3	1.6
L.F.D.B.	24	32	"	74.1	179.2	1.94	80.1	88	1.92	1.90	+3	+1	−6	−6	+2	−4	−1	+1	2.7
Gerald S.	25	18	"	45.2	171.8	1.48	1.47	1.49	1.50	1.49	−1	−1	−2	−2	−1	−1	0	+1	3.7
Fab. R.S.	26	18	"	32.7	141.5	1.11	1.28	1.15	1.15	1.19†	−1	−1	+8	+11	−4	+8	−3	−3	
R. H. S.	27	22	"	63.0	184.2	1.81	69.1	80	1.80	1.80	+1	−1	−7	−7	+1	−6	0	+1	
Mrs. McK.	28		F.	93.0	149.7	2.06	1.94	1.90	1.90	1.85	+8	+11	+5	+5	+11	+4	+2	−1	
Anna M.	29	1.8	"	6.25	73.20	0.40	0.85	0.36	0.34	0.37	+11	+17	(+130)*	(+150)*	+5	(+130)*	−3	−5	
Emma W.	30	26	"	57.6	164.8	1.63	1.59	1.60	1.61	1.65	+2	+1	−4	−1	−1	−4	−3	−2	
Average deviation for males and females											3.0	3.6	5.0	5.0	3.4	4.6	1.7	2.2	
" " "											1.3	1.0	5.7	5.7	2.0	4.8	1.3	1.6	
" " females											7.0	9.7	3.0	3.0	6.7	4.0	2.7	3.7	

* Figures in parentheses excluded from average.

† Adhesive plaster method = 3.3 per cent too high.

Subjects 1 to 20 are those published by Benedict in the *American Journal of Physiology*, 1916, xli, 289, Table 1.

Subjects 21 to 30 are those published by Du Bois and Du Bois in the *Archives of Internal Medicine*, 1916, xvii, 867, Table 3; *Archives of Internal Medicine*, 1916, xvii, 876, Table 4; Sawyer, Stone, and Du Bois, *Archives of Internal Medicine*, 1916, xvii, 862, Table 4.

The following statement of Benedict concerning the surface areas obtained by the photographic and by the Du Bois linear methods appears to us fully as applicable to a comparison between the surface areas obtained by the Du Bois height-weight formula and those calculated for normal adults by the formulas derived from the Harris and Benedict correlation formulas: "Comparisons between the photographed areas and the body surface as computed from the Du Bois linear formula show, even with the most diverse configurations of body, a constancy rarely observed in anatomical measurements or in computed ratios based upon such measurements."

A comparison similar to that for subjects shown in Table II may be made of the surface areas calculated by the Du Bois height-weight formula and by the formulas derived from Harris and Benedict's heat prediction formulas throughout the entire range of Harris and Benedict's tables. In Chart 1 are plotted curves for surface areas as calculated by the two methods for the extreme heights of 151 and 200 cm. between the ranges of 25 and 124 kilos for men and women. In Chart 2 are plotted the percentage variations of the Benedict curves from the Du Bois curves shown in Chart 1. From these curves it is evident that the greatest possible discrepancy between the two methods amounts to 19 per cent if restricted to the limits given in the Harris and Benedict tables, and occurs in men who are very fat and extremely short for their weight. Near this extreme range, however, there is a discrepancy of -11 per cent between the area calculated by the Du Bois height-weight formula from that by the Du Bois linear, while there is only a $+7$ per cent discrepancy between the area calculated by the Benedict formula for males from that by the Du Bois linear as illustrated by Subject 1 in Table II. If it is assumed that the linear formula and the photographic method are the most accurate methods, then the surface area obtained from Harris and Benedict's formula for men may, at these extreme ranges, predict the surface area with nearly the same accuracy as the Du Bois height-weight formula. Harris and Benedict, however, warn against the application of their formulas beyond the ranges given in their table, thus creating the suspicion that likewise the extreme ranges utilized are also open to question. The direction and position of their curves for women in relation

CHART 2.   Percentage variation of the surface areas as calculated by the derived Harris and Benedict formulas from that calculated by the Du Bois height-weight formula.   Curves A and B represent the percentage variation of the surface area as calculated by the Harris and Benedict formulas for men from that calculated by the Du Bois formula as shown in Chart 1.   Curve A is for a constant height of 200 cm. and Curve B is for a constant height of 151 cm.   Similarly for women, Curve D represents the percentage variation of Curve B from Curve A of Chart 1 (height 151 cm.) and Curve C the percentage variation of Curve C from Curve E of Chart 1 (height 200 cm.).   These curves illustrate the extreme limits of the percentage variation of the surface area as calculated by the formulas derived from the Harris and Benedict heat prediction formulas (for any height or weight included in their tables) from the surface area as calculated by the height-weight formula of Du Bois and Du Bois.

to their curves for men indicate why prolongation, especially of the formula for women, leads to improbable values for the surface area. It seems likely that that formula is best within limits which also allows with equal accuracy an extension beyond those limits. Chart 1 also reveals the peculiar fact that according to Harris and Benedict the value of the height-weight factor is for a woman 151 cm. in height and 25 kilos in weight, 9 per cent larger, while for a woman 200 cm. in height and 25 kilos in weight this is 2 per cent smaller than for a man of similar size; likewise for a woman 151 cm. in height and 124 kilos in weight, this height-weight factor is 9 per cent smaller, and for a woman of 200 cm. and 124 kilos in weight it is 17 per cent smaller than for a man of similar size. Harris and Benedict have as yet offered neither a reasonable explanation, nor sufficient experimental proof, to justify the embodiment of such a peculiar variation of the effect of height-weight on the heat production between men and women as to incorporate the same into a formula for the prediction of standards of heat production.

From these curves it is evident, however, that usually nearly the same values for variation in height and weight are utilized in the prediction of heat production in subjects of average adult size, both by the heat formulas of Harris and Benedict and the formulas of Du Bois and Du Bois, regardless of the theoretic considerations of surface area underlying the derivation of the formulas. Certain discrepancies in the relationship between the Harris and Benedict formulas for men and women as mentioned, however, indicate that the height-weight formula of Du Bois is of more general applicability.

On the other hand, an analysis of the predicted total calories for the effect of age reveals marked discrepancies in the value allotted to the age factor by Du Bois and by Harris and Benedict. By basing his comparisons for age on calories for each square meter of body surface Du Bois makes the same percentage decrease in heat production in a small or a large subject for increasing age; Harris and Benedict on the other hand subtract exactly the same number of calories for a given increase in age, regardless of the size of the subject. For example, as illustrated by Chart 3, the Du Bois method predicts a 10 per cent reduction in the basal heat production for a man 70 years of age, of any size, as

compared with a man of a similar height and weight at the age of
20 years; Harris and Benedict on the contrary predict that a large
subject (124 kilos, 200 cm.) will have a heat production 12.5 per

CHART 3. The percentage decrease in the heat production for increasing
age. Curve A for women and Curve D for men show the percentage
decrease in heat production for small subjects with heights of 151 cm. and
weights of 25 kilos, as predicted by Harris and Benedict; Curve B for women
and Curve E for men are the percentage decreases according to Harris and
Benedict in the heat production for advancing age, as predicted for large
subjects with a height of 200 cm. and a weight of 124 kilos. Curve C for
women and Curve F for men illustrate the percentage decrease in heat
production for increasing age, as predicted by the Du Bois normal stand-
ards. It is to be noted that according to Du Bois the size of the subject
does not alter the percentage decrease of the heat production for advancing
age, while according to Harris and Benedict a much greater percentage
decrease occurs in small than in large subjects.

cent less, and a small subject (25 kilos, 151 cm.) will have a heat production 32 per cent less at 70 years of age as compared with similar subjects 20 years of age. It does not appear to be in accordance with the facts, nor does it seem logical to assume, that a small man will show more than twice the percentage decrease in heat production for advancing age than a large man. The formula for women shows this same peculiarity (Chart 3) but to a lesser degree. We believe that the Du Bois age factor is more in accordance with the data at present available than that of Harris and Benedict.

In his normal standards Du Bois makes the same percentage difference in heat production for sex at any constant age, regardless of the size of the subject, because he bases his comparisons on calories for each square meter of body surface. In contrast, Harris and Benedict predict on the one hand a markedly lower heat production for large women than for similar sized men, but on the other hand they make the astounding prediction, which is contrary to their general conclusion, that the heat production of small women is greater than that of similar sized men. This peculiarity of their prediction formulas is illustrated in Chart 4, in which it is shown that, at the age of 21 years, a small woman (25 kilos, and 151 cm.) will have a heat production 5 per cent greater, and at the age of 70, a heat production 22 per cent greater than that of a man of similar height and weight at those respective ages. The assumption of such a reversed effect of sex on the heat production in small as opposed to large subjects does not appear to us to be sufficiently established by the data at present available to justify its incorporation into a prediction formula, particularly since theoretically it seems much more likely that both age and sex affect the heat production of large and small subjects in the same direction and probably also in approximately the same degree.

The larger discrepancies between the basal metabolic rate, as predicted from the formulas of Harris and Benedict and from those of Du Bois, result usually from a summation of the small discrepancies found for height and weight and the larger ones for age and sex; in some instances there is entire agreement as to the value of the different groups of factors, while in others the discrepancies approximately balance one another, thus accounting

for the parallelism between the basal metabolic rate calculated
by the Du Bois and by the Harris and Benedict standards, as
found by Boothby and Sandiford and by Means and Woodwell.

Constants for age and sex must always be used in the prediction
of the basal metabolic rate, either by proper standards for varying

CHART 4. Effect of sex on the heat production. Curve A represents
the percentage variation of the basal heat production of a large woman
(200 cm. and 124 kilos) from a similar sized man between the ages of 20 and
70 years, as predicted by Harris and Benedict. Curve B is the prediction
of Du Bois and is the same for all women, regardless of their size. Curve
C is the percentage base line. Curve D is the prediction of Harris and
Benedict for a small woman (151 cm. and 25 kilos) as compared to a similar
sized man.

age and sex as done by Du Bois, or by the introduction of the
constants directly into the formula as done by Harris and Bene-
dict. Certain critics of the Du Bois surface area normal standard
method neglect to emphasize the Du Bois standards for age and
sex and refer simply to surface area, and they so state their

arguments that to the superficial reader it may appear that Du Bois does not utilize the age and sex factors in the prediction of the normal basal metabolism. Such an implication is as unwarranted as it would be to refrain from using appropriately the four tables of Harris and Benedict one of which is for height and age in men, another for height and age in women, while the third and fourth are for the weight of men and women, respectively.

We, as well as all other recent writers, agree with Benedict that the rate of heat production is neither controlled by the area of the body surface, nor at the present stage of the evolution of man caused by the influence of cooling on the body; we also agree that in all probability the heat production is proportional to the active protoplasmic mass of the body for any given age and sex. However, as the protoplasmic mass of the body must be related to the total body nitrogen and since Moulton has shown that the surface area of beef cattle is a power function of the total body nitrogen, the formula being $S = N^{\frac{2}{3}}$, there is direct experimental evidence to the effect that the protoplasmic mass is proportional to the surface area. Furthermore, the constants of the height and weight as used by Harris and Benedict and by Du Bois and Du Bois, although derived by entirely different methods and from diametrically opposite theoretic considerations, lead to an estimation of the surface area in the average adult subject with practically equal accuracy. This remarkable fact seems to us, when taken in conjunction with the average demonstrated parallelism in the basal metabolic rate as calculated by the two methods, to be additional evidence that the surface area is the most exact method at present available for estimating the active protoplasmic mass and in consequence the best method, in conjunction with appropriate standards of age and sex, for predicting the basal heat production.

CONCLUSIONS.

1. It is shown that there is remarkable agreement between the surface area calculated by the Du Bois surface area formulas and the formulas derived from Harris and Benedict's biometric correlation formulas for the prediction of the basal heat production.

2. Since the factors for height and weight, as used by Harris and Benedict in their formulas for predicting the basal heat production, admit of the calculation of the surface area of subjects who have been measured by casts or Benedict's photographic method with approximately the same variation as that between the Du Bois height-weight formula and the Du Bois linear formula, the contention of Lusk and Du Bois is strengthened that the basal heat production, for a given age and sex, is proportional to the surface area.

3. As a marked difference exists between the Du Bois and the Harris and Benedict factors for age and sex, the major discrepancies between the basal metabolic rate as calculated from the two standards are due to the difference in values attributed thereto, especially when those of the latter are superimposed on the small differences obtained from the slightly different height-weight factors of the two methods.

4. Harris and Benedict, in their correlation formula, assume that a small subject will show more than twice the percentage decrease in heat production for advancing age than a large subject, while Du Bois assumes that age affects alike both small and large people.

5. Harris and Benedict assume a reversed action for sex, depending on the size of the subject, by predicting first that large men have a greater heat production than similar sized women and second that small women have a greater heat production than small men.

6. Until Harris and Benedict prove that the effect of age and of sex is different in large and small men and women, and that small women have a greater heat production than small men, it is best to adopt the factors of Du Bois which are in better accordance with the evidence at present available, namely that age and sex produce at least approximately the same percentage alteration in different subjects, regardless of their size.

7. The Du Bois formula for the determination of the surface area and the Du Bois normal standards of heat production for each square meter of body surface for age and sex are considered by us the best method at present available for predicting the normal heat production.

SUMMARY OF THE BASAL METABOLISM DATA ON 8,614 SUBJECTS WITH ESPECIAL REFERENCE TO THE NORMAL STANDARDS FOR THE ESTIMATION OF THE BASAL METABOLIC RATE.

By WALTER M. BOOTHBY and IRENE SANDIFORD.

(*From the Section on Clinical Metabolism, Division of Medicine, Mayo Clinic and Mayo Foundation, University of Minnesota, Rochester.*)

In the preceding paper we presented mathematical data which in our opinion indicate that the Du Bois normal standards based on calories for each square meter of body surface are the best so far advanced for predicting the basal heat production. We confirmed the previous observation of Du Bois that the height and weight factors as utilized by Harris and Benedict in their heat formulas predict the surface area in the average person with an approach to the same accuracy as the Du Bois height-weight formula. It was further pointed out that there was considerable discrepancy between the values for age and sex as assigned by Du Bois and by Harris and Benedict and that those adopted by the former were in better agreement with the evidence at present available.

The arguments advanced in that paper, however, need the support of further experimental evidence because, after all, standards cannot be maintained on mathematical considerations as opposed to experimental data. The object of this paper is to present in as brief a form as possible the basal metabolism data obtained in our laboratory from March, 1917, to January, 1922. During this time more than 25,000 basal metabolic rate determinations have been made on 8,614 subjects;[1] these results are

[1] Since going to press, an article by Means and Burgess has appeared (Means, J. H., and Burgess, H. W., *Arch. Int. Med.*, 1922, xxx, 507) in which they report metabolism studies on a series of 1,000 cases. Their data and conclusions are in accord with ours as to the clinical significance of alterations in the basal metabolic rate.

TABLE 1 *

Comparison of the Basal Metabolic Rate in 6,197 Patients with Thyroid Disorders.

Diagnosis.	Cases.	Percentage range.								
		Above + 20.	+ 20 to + 16	+ 20 to + 11	+ 15 to + 11	Normal + 10 to - 10.	- 11 to - 15	- 11 to - 20	- 16 to - 20	Below - 20.
		per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent
Exophthalmic goiter.......	2,569	93		5		2†				
Recurrent exophthalmic goiter‡................	320	90	6		2	2				
Adenoma with hyperthyroidism................	1,425	68		32§						
Recurrent adenoma with hyperthyroidism‡.......	46	57	17			26				
Adenoma without hyperthyroidism.............	1,111					100				
Recurrent adenoma without hyperthyroidism‡...	62					90	8		2	
Colloid goiter‖...........	328		3		10	79	6		1	1
Myxedema...............	102							20		80
Postoperative myxedema..	41							46		54
Questionable hypothyroidism....................	86					9		61		30
Cretinism...............	28					21		32		47
Thyroiditis.............	34	32			12	35		12		9
Malignant thyroid	45	22			9	67				2
Total No. of cases........	6,197									

* The 8,614 cases summarized in Tables I and II represent all patients on whom tests were made from March, 1917 to January, 1922 with the exception of 87 in which a positive diagnosis of the presence of hyperthyroidism was not reached. Two of the 87 patients had metabolic rates between -15 and -11 per cent; thirty-one between -10 and +10 per cent; thirty-five between +11 and +20 per cent; and nineteen above +20 per cent. About one-half of this group had but one metabolic rate determination.

In a consideration of the significance of Tables I and II, the probability of errors in the determination of the basal metabolic rate must be evaluated. We estimate that in routine work in our laboratory there is a material error in about 1 per cent of the determinations and in an additional 5 per cent

summarized in Tables I and II, and a few illustrative charts are given. The outstanding fact is that 77 per cent of all patients other than those with disorders of the thyroid had basal metabolic rates within the restricted Du Bois normal limits of $+10$ to -10 per cent; 90 per cent had basal metabolic rates within $+15$ to -15 per cent. The high percentage of normal results is most significant when it is considered that all, except 127 of the subjects who comprise our normal group, had a functional or organic disease.

Tables I and II include all patients on whom tests were made except the 87 referred to in the asterisk (*) footnote to Table I.

of the tests slight errors occur, the result of which is to place the patient either in the next higher or lower group, as arranged in the table. In about one-third of the patients with thyroid disorders only one determination was made and approximately one-half the patients who had other diseases, not involving the thyroid, had only one rate.

If in any group 100 typical cases with unquestioned diagnosis are selected and on whom sufficient basal metabolic rate determinations are made so that all errors are excluded, such a series will usually show a 99 per cent agreement with the characteristic metabolism for that condition.

† With a few exceptions the patients with exophthalmic goiter having basal metabolic rates below $+20$ per cent came under our observation during a period of remission.

‡ The patients listed under recurrent exophthalmic goiter and recurrent adenoma, with and without hyperthyroidism, include those who had had a previous partial thyroidectomy before any metabolism studies had been made in our laboratory and in whom the question of the necessity for further operative treatment was under consideration.

§ A basal metabolic rate of $+10$ per cent has been taken arbitrarily as dividing patients with adenomatous goiter into the groups with and without hyperthyroidism. While all cases with basal metabolic rates below $+10$ per cent are unquestionably not hyperthyroid, it cannot be assumed that all those with basal metabolic rates slightly above $+10$ per cent are necessarily hyperthyroid; unfortunately this group when tabulated was not subdivided at $+15$ per cent as was done in some of the other groups.

‖ The cases grouped in the table under colloid goiter include a considerable and unknown proportion of cases of colloid adenoma because in the earlier cases of the series less attention was directed to making a correct differential diagnosis than is at present exercised.

¶ Only rarely does a cretin come under our observation who has not had thyroid medication; therefore the results presented in the table cannot be considered as the average of a group of untreated cases. Furthermore, the normal standards for children are not yet as accurately established as are those for adults.

TABLE II.

The Basal Metabolic Rate in Conditions Not Due to Thyroid Disorders.

Diagnosis.	Cases.	Percentage range.							
		Below -20	-20 to -16	-15 to -11	-10 to +10	+11 to +15	+16 to +20	Above +20	-15 to +15
		per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent
Normals	127			3.2	92.1	4.0	0.7		99.3
Migraine	31			6.4	93.6				100.0
Chronic nervous exhaustion.	267		1.2	2.3	87.3	8.6	0.7		98.2
Neurasthenia	384		0.3	3.6	84.3	9.4	1.4	1.0	97.3
Obesity	94	1.1	3.2	6.4	80.7	7.5	1.1		94.6
Asthenia	36		2.7	11.2	77.8	8.3			97.3
Essential hypertension	170			0.6	73.0	15.8	7.2	3.4	89.4
Cardiac neurosis	99		1.0	2.0	83.9	10.1	1.0	2.0	96.0
Heart block	10				80.0	10.0		10.0	90.0
Endocarditis	56			1.8	80.4	5.4	1.8	10.6	87.6
Myocarditis	55	1.8		3.6	81.9	10.9		1.8	96.4
Pericarditis	4				100.0				100.0
Congenital heart	5				80.0	20.0			100.0
Renal	127	4.0	1.6	3.2	72.4	12.6	4.0	2.4	88.2
Hodgkin's disease	1							100.0	
Mental	34	3.0	3.0	8.9	61.7	17.6	2.9	2.9	88.2
Epilepsy	22	9.1	4.6	9.1	77.3				86.4
Gastrointestinal	98	1.0	1.0	3.1	85.7	4.1	5.1		92.9
Gynecological	96	1.0	2.1	6.3	81.3	4.1	5.2		91.7
Malignancy	0		5.0		55.0	10.0	10.0	20.0	65.0
Dermatological	43	2.3		14.0	79.1	4.6			97.7
Pregnancy	30				70.0	10.0	10.0	10.0	80.0
Encephalitis	10			10.0	70.0			20.0	80.0
Dysphagia	65	16.9	6.2	10.7	63.1	3.1			76.9
Acromegaly	30	3.3		3.3	43.4	13.3	10.0	26.7	60.0
Hypopituitarism	58	12.1	25.9	15.5	34.5	5.2	3.4	3.4	55.2
Paget's disease	6				66.7	16.7		16.6	83.4
Addison's disease	13	15.4		7.7	69.2			7.7	76.9
Polycythemia	2				50.0			50.0	50.0
Secondary anemia	30		3.3		80.0	13.4		3.3	93.4
Anemia, splenic and pernicious.	19			15.9	63.0	10.5		10.6	89.4
Leucemia, lymphatic and myelogenous	16				6.3	6.3		87.5	12.6
Questionable ductless glands.	24	8.3	8.3	16.6	58.4			8.4	75.0
Sclerosis of central nervous system; tabes	20				90.0	10.0			100.0
Diabetes	68	17.7	10.3	7.3	52.9		5.9	5.9	60.2
Arthritis	69	2.8	2.8	5.7	75.3	11.6	1.4		92.6
Miscellaneous, not thyroid.	178		2.7	5.6	77.1	8.4	4.0	2.2	91.1
Total No. of cases	2,117	2.1	2.2	4.6	77.1	8.3	2.6	3.1	90.0

In Table I are tabulated the basal metabolic rate findings in diseases of the thyroid and in Table II of all conditions other than of the thyroid, including such diseases as the leucemias which are characterized by abnormal basal metabolic rates. A study of these tables reveals most convincing evidence that the basal metabolic rate is characteristically normal in all except a few specific diseases.

In our laboratory, metabolic rates are obtained by the open or gasometer method with analysis of the expired air by the Haldane gas analysis apparatus. Original readings are made in duplicate by two observers. Two analyses are made of the expired air, and the results are accepted if they agree within 0.04 per cent for carbon dioxide and 0.06 per cent for oxygen; additional analyses are required if there is a greater discrepancy. The calculations are made by four place logarithms and checked by the nomographic chart method. Repeated outdoor air analyses, of which permanent records are kept, are made at stated intervals with each Haldane apparatus and by each analyst; similarly, analyses of a common sample of expired air are frequently made by all analysts. A very definite routine, described in detail in our laboratory manual has been adopted with the intent of reducing technical errors to a minimum. As a result we feel that accidental technical errors except those due to loss of expired air from an improper application of the face mask are of very rare occurrence. A material error from the cause mentioned occurs in approximately 1 per cent of the tests and always causes a lowering of the metabolic rate.

Technical errors are, however, a much less common source of inaccuracy than failure to obtain the metabolic rate under absolute *basal* conditions. A *basal* determination is not obtained if the subjects fail to cooperate, or are nervous and worried over the procedure as they are apt to be in the first test. Under such conditions the observed metabolic rate may be from 5 to 30 per cent too high. Therefore, a first test with an observed metabolic rate between +10 and +20 per cent will probably be lowered to within the normal limits of variation if the test is repeated a sufficient number of times to obtain a true basal metabolic rate. In the various groups reported in Table II, in which there are a few slightly elevated rates, experience has convinced us that

nearly all would have been normal if a sufficient number of tests had been made on those subjects to eliminate temporary (non-basal) elevations. These temporary elevations of the metabolic rate are, in clinical work by far the most confusing factor in the estimation of the significance of the basal metabolic rate and are of great importance in the consideration of data such as are presented in this paper. Nothing the subject does or fails to do, except deep sleep, can depress the metabolic rate, whereas any slight disturbing condition such as a headache, pain, discomfort, excitement, restless or uneasy sleep the preceding night, fear, movements, fever, surreptitious ingestion of food, or innumerable other factors may, in certain cases, cause an appreciable elevation.

The cause of the temporary elevation in the metabolic rate is not definitely known aside from that due to fever, food, and muscular movement. Aub has pointed out that with the exception of the thyroid and adrenal glands there is at present no evidence that any other ductless gland produces a demonstrable calorigenic reaction. Plummer and Boothby have shown that these rapid fluctuations in the metabolic rate cannot be accounted for in man by corresponding variations in the thyroxin concentration in the tissues because the maximum effect of a single intravenous dose of 10 mg. of thyroxin is not usually reached until between the 5th and 10th day after administration and it takes as a rule several weeks for the reaction entirely to disappear. In some instances these temporary elevations are probably due, as suggested by Aub, to the emotional stimulation of the adrenal glands resulting in a discharge into the circulation of an excess of adrenalin secretion. This explanation is in accordance with our observations as we have shown that adrenalin, when injected into dogs in quantities within the accepted power of the adrenal glands to secrete, produces a definite calorigenic reaction; the alteration in heat production under such conditions is an increase of from about 5 to 20 per cent, which is of the same average order of magnitude and duration noted in these temporary elevations met with occasionally in clinical work. Elevations of this type in the observed rate must be carefully differentiated from true elevations of the *basal* metabolic rate.

In Chart 1 are plotted the basal metabolic rates, computed from the Du Bois surface area standards, obtained on 127 subjects whom we consider "normal" because they had passed through a

CHART 1.

CHART 2.

careful clinical examination without revealing any evidence of disease that could in any way affect their metabolism. The criticism that these subjects were practically all "patients" and came for examination because they thought they were, or might be, ill is effectively offset by this careful negative examination; several of the subjects had bad teeth, tonsils, a hernia, or other mechanical fault, all of which, as is recognized, could in no way affect the metabolism. Of the 127 subjects plotted on the chart, 109 had only one metabolic rate determination; none of those with results between $+11$ and $+15$ per cent or between -11 and -15 per cent, and only eighteen of those between $+10$ and -10 per cent had more than a single test. In Chart 2 are plotted the basal metabolic rates, computed from the Harris and Benedict standards, of the 102 subjects in Chart 1 who are 21 years, or more, or age.

In Table III are given the essential data with regard to this group of 102 normal subjects who are 21 years, or more, of age. The most interesting evidence in this table is the fact that the surface areas agree in most instances within 1 per cent, as calculated by the Du Bois height-weight formula and by the Harris and Benedict formulas derived according to the method given in the preceding paper; in only one case does the surface area as calculated by the two methods differ by as much as 5 per cent. The variations in the height-weight factors are in all cases so slight that the maximal alteration in the metabolic rate from the difference in value of these factors expressed as surface area is never more than 6 points from that calculated by the Du Bois height-weight factors for surface area, and is usually very much less. On the other hand, considerable variation is caused in many instances by the difference in value between the two standards for the factors of age and sex. These variations in the age and sex factors develop differences of at least 5 and possibly 14 points in the basal metabolic rate in a considerable proportion of the cases. The basal metabolic rates, as calculated by both methods, however, are in agreement when there is a cancellation of the variations of height and weight by the age and sex factors, or when all factors of both methods are of practically equal value.

We have also calculated a table similar to Table III, using the data reported by Harris and Benedict (their Tables C and D) for their 136 normal men and 103 normal women. The calcu-

TABLE III.

Essential Data on 102 Normal Persons.

No.	Sex.	Age.	Height	Weight.	Calories for each 24 hrs.	Surface area.		Basal metabolic rate.	
						Du Bois* height-weight formula.	Harris and Benedict formula.	Du Bois standards.	Harris and Benedict standards.
			cm.	*kg.*		*sq. m.*	*sq. m.*	*per cent*	*per cent*
1	F.	21	156.5	55.6	1,435	1.54	1.55	+5	+4
2	"	21	164.5	48.0	1,483	1.50	1.49	+11	+12
3	"	22	162.0	64.6	1,354	1.68	1.66	−9	−8
4	"	22	153.5	47.0	1,330	1.42	1.45	+5	+3
5	"	22	160.6	73.9	1,612	1.77	1 76	+3	+4
6	"	22	159.5	51.2	1,439	1.51	1.51	+7	+8
7	"	23	170.0	56.1	1,390	1.64	1.59	−4	−1
8	"	24	166.7	83.9	1,641	1.93	1.88	−4	−1
9	"	24	157.9	57.7	1,274	1.57	1.58	−8	−8
10	"	24	155.5	67.2	1,529	1.66	1.68	+4	+4
11	"	25	161.9	46.5	1,294	1.46	1.47	0	+1
12	"	25	154.6	37.0	1,303	1.28	1.35	+15	+11
13	"	25	158.6	69.1	1,461	1.70	1.70	−3	−2
14	"	25	165.6	57.5	1,342	1.64	1.59	−8	−4
15	"	27	164 0	45.6	1,330	1.46	1.46	+3	+5
16	"	27	167.5	60.0	1,551	1.66	1.62	+5	+10
17	"	27	168.5	65.3	1,598	1.73	1.68	+4	+9
18	"	27	154 4	67.5	1,434	1.66	1.68	−3	−2
19	"	28	163 7	68.6	1,461	1.75	1.71	−6	−1
20	"	30	164.4	66.1	1,466	1.72	1.68	−3	+1
21	"	31	155.5	66 8	1,428	1.65	1.67	−1	−1
22	"	32	163.9	54.8	1,248	1.58	1.56	−10	−6
23	"	32	166.3	55.0	1,410	1.61	1.57	0	+5
24	"	32	152.0	49.0	1,191	1.43	1.47	−5	−5
25	"	33	161.1	49.5	1,450	1.50	1.50	+10	+14
26	"	33	164.9	69.8	1,485	1.77	1.72	−4	+1
27	"	33	157 0	45.3	1,233	1.42	1.44	−1	+1
28	"	34	165 0	52 8	1,500	1.56	1.54	+10	+15
29	"	34	161.6	69.2	1,417	1.73	1.71	−7	−3
30	"	35	162.0	53.4	1,343	1.55	1.54	−1	+3
31	"	36	153.9	63.8	1,448	1.61	1.64	+3	+5
32	"	36	161.1	56.3	1,295	1.58	1.57	−7	−2
33	"	36	163.0	64.0	1,597	1.68	1.66	+8	+14
34	"	36	172.0	92.1	1,818	2.04	1.98	+2	+8
35	"	37	160.6	61.7	1,367	1.64	1 63	−5	0

* The Du Bois surface area is obtained by using a nomographic chart and, therefore, may vary from a mathematically calculated area by approximately 1 per cent.

TABLE III—*Continued.*

No.	Sex.	Age.	Height.	Weight.	Calories for each 24 hrs.	Surface area.		Basal metabolic rate.	
						Du Bois height-weight formula	Harris and Benedict formula.	Du Bois standards.	Harris and Benedict standards.
			cm.	*k*.		*sq. m.*	*sq. m.*	*per cent*	*per cent*
36	F.	37	156.0	46.2	1,264	1.42	1.45	+2	+4
37	"	37	167.5	56.9	1,408	1.63	1.59	−1	+5
38	"	39	160.0	59.3	1,386	1.60	1.60	−1	+4
39	"	39	160.1	64.4	1,493	1.67	1.65	+2	+8
40	"	39	154.5	60.6	1,302	1.59	1.60	−7	−3
41	"	39	159.8	55.1	1,382	1.55	1.55	+2	+7
42	"	39	154.5	55.0	1,377	1.52	1.54	+4	+7
43	"	39	153.0	55.5	1,333	1.52	1.54	0	+4
44	"	41	165.1	61.8	1,386	1.66	1.64	−3	+2
45	"	42	151.8	76.5	1,552	1.73	1.77	+4	+5
46	"	43	176.6	92.2	1,712	2.08	1.99	−5	+3
47	"	43	153.5	49.4	1,239	1.45	1.48	−1	+2
48	"	46	163.3	64.9	1,467	1.70	1.67	0	+8
49	"	46	168.7	86.9	1,651	1.97	1.92	−3	+4
50	"	48	163.8	68.0	1,401	1.74	1.70	−7	+1
51	"	50	165.8	74.5	1,512	1.82	1.78	−1	+5
52	"	50	160.0	52.7	1,411	1.53	1.53	+10	+16
53	"	52	165.5	90.0	1,601	1.98	1.94	−4	+1
54	"	52	159.0	73.3	1,510	1.76	1.75	+2	+7
55	"	52	162.0	44.7	1,197	1.44	1.45	−1	+5
56	"	53	151.0	51.7	1,289	1.45	1.50	+6	+9
57	"	53	165.7	47.4	1,397	1.50	1.48	+11	+20
58	"	55	160.3	46.4	1,136	1.46	1.46	−7	0
59	"	55	166.2	67.0	1,402	1.73	1.70	−3	+4
60	"	62	158.5	47.3	1,267	1.45	1.47	+7	+14
61	"	67	154.1	45.9	1,246	1.41	1.44	+8	+17
62	M.	22	168.3	66.3	1,849	1.76	1.76	+11	+11
63	"	23	173.1	61.9	1,822	1.74	1.73	+10	+12
64	"	23	177.7	76.9	1,800	1.95	1.97	+1	+1
65	"	23	168.7	67.6	1,647	1.77	1.79	−2	−2
66	"	23	180.0	64.6	1,894	1.82	1.80	+10	+11
67	"	24	168.9	59.2	1,688	1.67	1.67	+7	+8
68	"	24	172.0	54.2	1,570	1.63	1.61	+2	+4
69	"	25	171.0	58.0	1,692	1.68	1.66	+6	+9
70	"	25	180.2	75.8	1,963	1.96	1.96	+6	+7
71	"	25	178.3	70.5	1,904	1.88	1.88	+7	+8
72	"	27	165.0	61.3	1,664	1.66	1.68	+6	+7

TABLE III—*Concluded*.

No.	Sex.	Age.	Height	Weight	Calories for each 24 hrs.	Surface area.		Basal metabolic rate.	
						Du Bois height-weight formula.	Harris and Benedict formula	Du Bois standards.	Harris and Benedict standards.
			cm.	kg.		sq. m.	sq. m.	per cent	per cent
73	M.	27	173.4	56.7	1,644	1.68	1.65	+3	+8
74	"	27	171.0	62.0	1,675	1.73	1.72	+2	+5
75	"	28	171.8	70.4	1,773	1.81	1.85	+3	+4
76	"	29	161.3	64.9	1,588	1.67	1.71	0	+1
77	"	31	167.6	57.8	1,595	1.66	1.64	+1	+7
78	"	31	169.0	68.2	1,524	1.77	1.80	−9	−7
79	"	32	170.9	60.8	1,679	1.71	1.70	+4	+9
80	"	33	162.5	68.0	1,680	1.72	1.76	+3	+5
81	"	34	177.7	66.9	1,588	1.83	1.83	−8	−4
82	"	35	175.0	61.0	1,656	1.73	1.72	+1	+7
83	"	35	173.2	66.0	1,745	1.79	1.79	+3	+9
84	"	35	172.2	70.5	1,748	1.82	1.85	+1	+5
85	"	35	173.1	70.4	1,658	1.84	1.85	−5	0
86	"	37	172.9	67.8	1,680	1.81	1.81	−2	+4
87	"	38	164.0	54.6	1,524	1.58	1.57	+2	+10
88	"	39	170.0	82.0	1,776	1.92	2.00	−2	0
89	"	39	172.0	51.8	1,382	1.60	1.58	−9	+1
90	"	39	180.8	72.7	1,827	1.92	1.93	0	+7
91	"	40	180.0	89.9	1,854	2.09	2.17	−4	−4
92	"	43	172.3	70.2	1,773	1.83	1.84	+5	+11
93	"	44	181.0	71.3	1,830	1.91	1.91	+4	+11
94	"	45	167.1	67.5	1,593	1.75	1.78	−2	+4
95	"	47	175.0	68.8	1,908	1.84	1.84	+12	+21
96	"	51	177.4	62.0	1,404	1.78	1.75	−13	−4
97	"	54	178.4	78.8	1,535	1.97	2.00	−13	−8
98	"	57	182.6	67.0	1,730	1.87	1.85	+3	+14
99	"	58	169.0	58.2	1,393	1.65	1.65	−6	+5
100	"	58	154.3	60.8	1,526	1.58	1.61	+7	+19
101	"	58	179.0	78.0	1,690	1.97	1.99	−5	+3
102	"	60	169.5	53.2	1,445	1.61	1.59	+2	+16

lation of the surface area by the formulas derived from the Harris and Benedict heat prediction formulas gives on their own subjects a figure almost identical with the surface area obtained by the Du Bois height-weight formula. Space prevents the reproduction of this table but it can be summarized as follows:

The average surface area for the 136 normal men according to the Du Bois height-weight factors is 1.76 square meters and according to the Harris and Benedict height-weight factors for men is also 1.76 square meters; for the 103 normal women the Du Bois factors give an average of 1.59 square meters and the Harris and Benedict factors for women, 1.57 square meters; the

TABLE IV.

Summary by Decades of the Essential Data on 102 Normal Persons.

Decade.	Total No of cases.	Height.	Weight.	Surface area.		Basal metabolic rate.	
				Du Bois.	Harris and Benedict.	Du Bois.	Harris and Benedict.
Females.							
		cm.	kg.	sq. m.	sq. m.	per cent	per cent
21–29	19	161.3	59.1	1.61	1.60	+0.9	+2.3
30–39	24	160.5	59.3	1.61	1.60	−0.4	+3.6
40–49	7	163.4	71.4	1.76	1.74	−2.1	+3.6
50–59	9	161.8	60.9	1.63	1.62	+1.4	+7.4
60–69	2	156.5	46.6	1.43	1.46	+7.5	+15.5
Average..................	61	161.2	60.4	1.62	1.61	+0.3	+4.2
Males.							
21–29	15	172.0	64.7	1.76	1.76	+4.8	+6.3
30–39	14	171.6	65 6	1.76	1.77	−1.4	+3.8
40–49	5	175.0	73.5	1.88	1.91	+3.0	+8.6
50–59	6	173.5	67.5	1 80	1.81	−4.5	+4.8
60–69	1	169.5	53.2	1.61	1 59	+2.0	+16.0
Average..................	41	172.4	66.2	1.78	1.79	+1.0	+5.7
Average for males and females..................	102	165.7	62.8	1.69	1.68	+0.6	+4.8

individual variations are of approximately the same order of magnitude as shown in our own series given in Table III.

In Table IV is given the grand average of the 102 normal persons, and the average for each decade according to sex. The average surface area for the entire group calculated by the Du Bois height-weight formula is 1.69 square meters and by the

Harris and Benedict factors, 1.68 square meters, a negligible variation of 0.6 per cent. The average basal metabolic rate by the Du Bois method is +0.6 per cent and by the Harris and Benedict +4.8 per cent, again showing that the main variation in the results is due to a discrepancy in the age-sex factors and not to a fundamental disagreement in the values for the factors of height and weight.

CHART 3.

On Chart 3 are plotted the basal metabolic rates according to the Du Bois method for thirty-one patients who had severe migraine, but were otherwise entirely normal. It shows an even better percentage of cases falling within the Du Bois limits of normality and only eight had more than one rate. On Chart 4 are plotted the basal metabolic rates of 267 patients with chronic nervous exhaustion, and on Chart 5 the rates of 384 patients with neurasthenia. These two groups are essentially similar, as some of our clinicians prefer one term and some the other, although as a rule under the latter term are included the more easily excitable patients. This difference in temperament is illustrated by the tendency for the first metabolism test to be slightly higher in the latter group; only about one-fourth of these patients had

CHART 4.

CHART 5.

CHART 6.

CHART 7.

CHART 8.

CHART 9.

more than one rate.   Chart 6 illustrates the tendency of a single
clinical determination in certain persons with cardiac neurosis to
be above the present Du Bois normal standards, while the rates in
obesity, as shown in Chart 7, show an opposite tendency.   Charts 8
and 9 serve to illustrate the metabolic rate findings in patients
with gynecologic and gastrointestinal diseases.

TABLE V.

*Comparison of the Surface Area and Basal Metabolism as Calculated by the
Du Bois and by the Harris and Benedict Methods in 455 Persons.*

Sex.	Total No. of cases.	Height.	Weight.	Surface area.		Basal metabolic rate.	
				Du Bois.	Harris and Benedict.	Du Bois.	Harris and Benedict.
Chronic nervous exhaustion.							
		cm.	*kg.*	*sq. m.*	*sq. m.*	*per cent*	*per cent*
Females.....................	222	162.4	53.8	1.56	1.55	+1.2	+5.0
Males.....................	27	172.6	60.6	1.71	1.71	+1.0	+5.7
" and females	249	163.5	54.6	1.57	1.56	+1.2	+5.1
Migraine.							
Females.....................	27	161.3	55.2	1.56	1.56	−1.0	+3.1
Males...	2	174.0	66.0	1.79	1.79	+3.5	+12.5
" and females........	29	162.1	56.0	1.58	1.57	−0.7	+3.8
Obesity.							
Females.....	61	161.9	96.1	1.99	2.00	−0.8	+1.5
Males	12	173.3	105.3	2.17	2.36	+0.1	−3.9
" and females........	73	163.7	97.6	2.02	2.06	−0.7	+0.5
Normals.							
Females.....................	61	161.2	60.4	1.62	1.61	+0.3	+4.2
Males.....................	41	172.4	66.2	1.78	1.79	+1.0	+5.7
" and females........	102	165.7	62.8	1.69	1.68	+0.6	+4.8

Tables similar to Tables III and IV have been made for the
cases of chronic nervous exhaustion (249), migraine (twenty-nine),
and uncomplicated obesity (73) that fall within the limits of the
Harris and Benedict tables.   It is impracticable to publish these
in full; therefore, only the averages for the males and females are
given in Table V.   The surface area for any group is almost

TABLE VI.

Comparison of the Basal Metabolic Rate as Calculated by the Du Bois and by the Harris and Benedict Methods in 455 Persons.

		Total cases	Below −20	−20 to −16	−15 to −11	−10 to −6	−5 to +5	+6 to +10	+11 to +15	+16 to +20	Above +20	−10 to +10	−15 to +15
			per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent
Chronic nervous exhaustion.													
Du Bois.	Female	222		1	3	13	52	23	7	0.5		88	98
	Male	27			7	15	52	7	19			74	100
	" and female	249		1	4	13	52	21	8	0.4		87	98
Harris and Benedict.	Female	222		0.5	2	5	45	25	17	5	1	74	93
	Male	27				11	26	33	22	7		70	93
	" and female	249		0.4	2	6	43	26	18	6	1	74	93
Migraine.													
Du Bois.	Female	27			7	19	56	19				93	100
	Male	2					100					100	100
	" and female	29			7	17	59	17				93	100
Harris and Benedict.	Female	27				15	52	19	15			85	100
	Male	2						100				0	100
	" and female	29				14	48	17	21			79	100
Obesity.													
Du Bois.	Female	61	2	3	7	11	52	16	8			80	95
	Male	12			8	25	50	8	8			83	100
	" and female	73	1	3	7	14	52	15	8			81	96
Harris and Benedict.	Female	61		2	8	8	46	20	15	2		74	97
	Male	12			17	17	58	8				83	100
	" and female	73		1	10	10	48	18	12	1		75	97
Normals.													
Du Bois.	Female	61				16	66	13	5			95	100
	Male	41			5	10	61	19	5			90	100
	" and female	102			2	14	64	16	5			93	100
Harris and Benedict.	Female	61				5	64	16	10	5		85	95
	Male	41				5	44	29	15	5	2	78	93
	" and female	102				5	56	22	12	5	1	82	94

In the calculations the percentages were carried to two decimal places. In the table the nearest round numbers have been used, thus explaining apparent slight discrepancies in the addition of the percentages.

identical, whether calculated by the Harris and Benedict or the Du Bois formula. The least variation in the basal metabolic rate is in the group of obese persons, because, as shown in the previous paper, the age and sex factors of Du Bois and of Harris and Benedict are in much closer agreement for large than for small subjects. In Table VI are given the percentages of subjects who have basal metabolic rates within certain ranges as calculated by the Du Bois and by the Harris and Benedict methods. A study of this table reveals the fact that the basal metabolic rates, as calculated by the Du Bois method, are more often between -5 and $+5$ per cent, between -10 and $+10$ per cent, and between -15 and $+15$ per cent than by the Harris and Benedict method.

As we have said, if repeated tests were made a considerable number of the determinations plotted in the charts above the zero line would be from 1 to 5 points lower; while a smaller proportion of those below that line would be lowered but less on the average. There is some evidence to indicate that possibly the absolute level of the Du Bois age and sex standards is slightly too high; however, at the present time there are not sufficient data available to warrant their alteration. In our opinion the correlation study of Harris and Benedict has confirmed the accuracy of the Du Bois height-weight factors independently of any theoretic considerations with regard to surface area so that the next improvement to be anticipated is in the age-sex factors.

SUMMARY.

The charts and tables show the high percentage of persons who have normal basal metabolic rates according to the Du Bois standards, unless they are suffering from some specific disease characterized by an alteration in the basal metabolism. The limitations of the basal metabolic rate as a diagnostic aid can likewise be evaluated. As the clinical significance of abnormal basal metabolic rates will be treated in detail in other publications, we shall not here discuss this phase of the subject, except to point out that the data here presented are evidence to the effect that the basal metabolic rate differentiates diseases into those with increased, normal, and decreased metabolism as sharply as the temperature divides diseases into the febrile and afebrile groups.

The two points we wish especially to emphasize are; first, that a high percentage of persons has a basal metabolic rate within ± 10 per cent and a very high percentage within ± 15 per cent of the Du Bois standards for age and sex for each square meter of body surface provided the subjects have no definite disease that is characterized by a pathologic alteration in the rate of heat production; and second, that a smaller percentage of these same subjects has basal metabolic rates within the same limits when the Harris and Benedict standards are used.

CONCLUSION.

The Du Bois normal standards for age and sex based on calories for each square meter of body surface determined by the Du Bois height-weight formula are the best standards at present available for the prediction of the normal heat production, as shown by a study of the basal metabolic rate of 8,614 persons.

BIBLIOGRAPHY.

Aub, J. C., and Du Bois, E. F., Clinical calorimetry. Nineteenth paper. The basal metabolism of old men, *Arch. Int. Med.*, 1917, xix, 823.

Aub, J. C., The relation of the internal secretions to metabolism, *J. Am. Med. Assn.*, 1922, lxxix, 95.

Aub, J. C., and Taylor, M., The effect of body tissues other than the thyroid upon the basal metabolic rate, *Endocrinol.*, 1922, vi, 255.

Benedict, F. G., A photographic method for measuring the surface area of the human body, *Am. J. Physiol.*, 1916, xli, 275.

Benedict, F. G., The relationship between body surface and heat production especially during prolonged fasting, *Am. J. Physiol.*, 1916, xli, 292.

Boothby, W. M., and Sandiford, I., Laboratory manual of the technic of basal metabolic rate determinations, Philadelphia, 1920, 15.

Boothby, W. M., The basal metabolic rate in hyperthyroidism, *J. Am. Med. Assn.*, 1921, lxxvii, 252.

Boothby, W. M., The fundamental classification of disease by the basal metabolic rate, *J. Am. Med. Assn.*, 1921, lxxvi, 84.

Boothby, W. M., Diagnosis and treatment of the diseases of the thyroid gland, New York, 1921, iii, 833.

Boothby, W. M., and Sandiford, R. B., Nomographic charts for the calculation of the metabolic rate by the gasometer method, *Boston Med. and Surg. J.*, 1921, clxxxv, 337.

Boothby, W. M., and Sandiford, I., The relationship of the increase in blood sugar concentration to the specific dynamic action of glucose and to the specific dynamic action of adrenalin, *Am. J. Physiol.*, 1921, lv, 293.

Boothby, W. M., and Sandiford, I., Calorigenic effect of adrenalin in dogs, *Am. J. Physiol.*, 1922, lix, 463.

Boothby, W. M., and Sandiford, I., A comparison of the Du Bois and the Harris and Benedict normal standards for the estimation of the basal metabolic rate, *J. Biol. Chem.*, 1922, liv, 767.

Compilation on basal metabolism, its determination and application, Boston, 1922, 29.

Du Bois, D., and Du Bois, E. F., The measurement of the surface area of man, *Arch. Int. Med.*, 1915, xv, 868.

Du Bois, D., and Du Bois, E. F., Clinical calorimetry. Tenth paper. A formula to estimate the approximate surface area if height and weight be known, *Arch. Int. Med.*, 1916, xvii, 863.

Du Bois, E. F., Clinical calorimetry. Twelfth paper. The metabolism of boys 12 and 13 years old compared with the metabolism at other ages, *Arch. Int. Med.*, 1916, xvii, 887.

Du Bois, E. F., The basal metabolism in fever, *J. Am. Med. Assn.*, 1921, lxxvii, 352.

Du Bois, E. F., "Basal metabolism"—a disclaimer of responsibility, *J. Am. Med. Assn.*, 1922, lxxviii, 916.

Gephart, F. C., and Du Bois, E. F., Clinical calorimetry. Thirteenth paper. The basal metabolism of normal adults with special reference to surface area, *Arch. Int. Med.*, 1916, xvii, 902.

Harris, J. A., and Benedict, F. G., A biometric study of human basal metabolism, *Proc. Nat. Acad. Sc.*, 1918, iv, 370.

Harris, J. A., and Benedict, F. G., A biometric study of basal metabolism in man, *Carnegie Inst. Washington, Pub. 279*, 1919.

Lusk, G., Fundamental ideas regarding basal metabolism, *J. Am. Med. Assn.*, 1921, lxxvii, 250.

Means, J. H., and Woodwell, M. N., Remarks on standards for normal basal metabolism, *Arch. Int. Med.*, 1921, xxvii, 608.

Moulton, C. R., Units of reference for basal metabolism and their interrelations, *J. Biol. Chem.*, 1916, xxiv, 299.

Murlin, J. R., On the law of surface area in energy metabolism, *Science*, 1921, liv, 196.

Sandiford, I., The effect of the subcutaneous injection of adrenalin chlorid on the heat production, blood pressure and pulse rate in man, *Am. J. Physiol.*, 1920, li, 407.

Sawyer, M., Stone, R. H., and Du Bois, E. F., Clinical calorimetry. Ninth paper. Further measurements of the surface area of adults and children, *Arch. Int. Med.*, 1916, xvii, 855.

ON DIACETONE GLUCOSE.

By P. A. LEVENE and G. M. MEYER.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

In a previous paper[1] it was reported that diacetone glucose and monoacetone benzylidene glucose on treatment with phosphorus oxychloride yield two phosphoric esters, which seem to differ one from another by the allocation of the phosphoric acid radical.

It was mentioned in this publication that additional and, if possible, more direct evidence of the structure of diacetone glucose and of acetone benzylidene glucose is desirable before a final discussion of the differences in the two phosphoric esters.

Two views have been advanced regarding the structure of diacetone glucose. According to the original view of Irvine and Scott,[2] the respective positions of the substituting groups were 1, 2 and 3, 5. Later, Macdonald,[3] in Irvine's laboratory, revised the theory, ascribing to the second substituting group, the position 5, 6. Karrer and Hurwitz,[4] on the basis of very imperfect experimental evidence, came to the same conclusion. On the other hand, Irvine and Hogg[5] have later reversed the conclusion of Macdonald and attributed to the second substituting group the position 3, 5. Thus, at present, the two views are still under discussion.

The present communication contains direct evidence in favor of the theory that in diacetone glucose the positions of the acetone radicals are on carbon atoms 1, 2 and 5, 6.

[1] Levene, P. A., and Meyer, G. M., *J. Biol. Chem.*, 1922, liii, 431.

[2] Irvine, J. C., and Scott, J. P., *J. Chem. Soc.*, 1913, ciii, 568.

[3] Macdonald, J. L. A., *J. Chem. Soc.*, 1913, ciii, 1896.

[4] Karrer, P., and Hurwitz, O., *Helvetica Chim. Acta*, 1921, iv, 728.

[5] Irvine, J. C., and Hogg, T. P., *J. Chem. Soc.*, 1914, cv, 1386.

According to the theory of Irvine and Hogg[5] the methylation of diacetone glucose should yield 6-methyl glucose. This on oxidation should yield 6-methyl gluconic acid, whereas according to the other theory 3-methyl saccharic acid should be obtained. On oxidation of the methyl glucose prepared by us 1,4-anhydro-3-methyl saccharic acid of the following structure, was obtained.

The reasons which led Irvine and Hogg to a different conclusion undoubtedly lie in the fact that they failed to obtain the oxidation product of monomethyl glucose in crystalline form.

Work on the acetone benzylidene glucose is in progress.[6]

EXPERIMENTAL.

3-Methyl Glucose.—30 gm. of diacetone, which were prepared as previously described, were methylated with an excess of dimethyl sulfate by the method of Haworth,[7] using 150 cc. of 30 per cent sodium hydroxide and 90 cc. of dimethyl sulfate. The temperature of the bath was kept at 70°C. The product of methylation was extracted with ether, the ethereal solution dried with anhydrous sodium sulfate, and the ether removed under diminished pressure. The colorless syrup was converted into the free sugar. For this purpose it was dissolved (25 gm.) in 400 cc. of 50 per cent alcohol containing 1.6 gm. of HCl, and heated in boiling water with reflux for 70 minutes. The acid was removed with silver carbonate, and the excess of silver with H_2S. The filtrate was decolorized with charcoal and concentrated to a syrup under diminished pressure without heat. The syrup was

---

[6] At the proof-reading of this communication, the latest publication of Irvine and Patterson (Irvine, J. C., and Patterson, J., *J. Chem. Soc.*, 1922, cxxi, 2146) has reached us. In that publication they attribute to the diacetone glucose the structure of a γ-glucose. If that contention is further corroborated, the position 4 and not 3 will have to be assigned to the methyl group.

[7] Haworth,W. N., *J. Chem. Soc.*, 1915, cvii, 8.

taken up in a small amount of dry methyl alcohol and allowed to crystallize in an ice-alcohol cooling mixture. The crude product was recrystallized several times from methyl alcohol. The substance was identical with that described by Irvine and Scott.[2] It melted at 158°. It had the following final optical rotation in water:

$$[\alpha]_D^{20} = \frac{+1.14° \times 100}{1 \times 2} = +57°$$

The substance analyzed as follows:

0.1030 gm. substance: 0.1638 gm. CO_2 and 0.0684 gm. H_2O.
$C_7H_{14}O_6$.   Calculated.   C 43.30, H 7.22.
Found.   " 43.36, " 7.43.

1,4-Anhydro-3-Methyl Saccharic Acid.—2 gm. of methyl glucose were dissolved in 50 cc. of 50 per cent nitric acid and allowed to stand at room temperature for 42 hours. The solution was then divided onto two clock-glasses and rapidly evaporated on a boiling water bath to complete dryness. This was moistened with 5 cc. of 50 per cent nitric acid and again evaporated to dryness. The residue was then evaporated twice with 5 cc. of water.

The dry material was taken up in acetone and ether from which it was obtained crystalline. It sintered and turned dark at 190° and melted at 206–207°C.

The substance had the following optical rotation:

$$[\alpha]_D^{20} = \frac{+0.15° \times 100}{0.5 \times 2} = +15°$$

The substance analyzed as follows:

0.1077 gm. substance: 0.1612 gm. CO_2 and 0.0490 gm. H_2O.
$C_7H_{10}O_7$.   Calculated.   C 40.76, H 4.89.
Found.   " 40.81, " 5.09.

0.1006 gm. of substance dissolved in water required for immediate titration 5.4 cc. of 0.1 N alkali (phenolphthalein as indicator). On allowing this solution to stand for 1 hour with an excess of 0.1 N NaOH, 10.2 cc. of 0.1 N alkali were utilized. The theory for the lactone requires 4.85 cc. of 0.1 N alkali and for the dibasic acid 9.70 cc. of 0.1 N alkali.

REDUCTION OF BENZYLIDENE-1-ETHYL-2-DIAZOGLUCONATE.

By P. A. LEVENE.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

It was previously reported that benzylidene-1-ethyl-2-diazogluconate, on treatment with acetic acid, with dry hydrogen chloride, and with dry hydrogen bromide, is transformed, respectively, into the benzylidene esters of gluconic, chloromannonic, and bromomannonic acids.[1] In no instance could the epimers of these substances be detected. In the present communication are reported the reduction experiments with aluminum amalgam. Two products are obtained as a result of this reaction; one is benzylidene-ethylchitosaminate and the other 5,6-benzylidene-1-ethyl-2-desoxygluconate (mannonate). The formation of the chitosamine derivative took place apparently asymmetrically, as the crude product, judging from its optical rotation, contained no traces of its epimer. The reaction proceeded in the following way:

The desoxygluconic derivative was found identical with the product obtained earlier by the reduction of 5,6-benzylidene-1-

[1] Levene, P. A., *J. Biol. Chem.*, 1922, liii, 449.

ethyl-2, 3-anhydromannonate.   The formation of the two products in our experiments is noteworthy in connection with the experience of Staudinger and his coworkers.[2] On reduction of diazoacetic ester with aluminum amalgam they observed the formation of only one product; namely, glycine ester.   On the other hand, on reduction with hydrogen gas in the presence of palladium, only ethyl acetate was formed.

As regards the mechanism of the formation of the desoxy derivative the following possibility had to be considered.   It had been mentioned[1] that the same substance was formed on the reduction of the anhydro derivative of the following composition:

$$CH_2 \cdot CH \cdot CH\ OH \cdot CH\ OH \cdot COH = CH \cdot COOC_2H_5 *$$

with the structure showing an O–O bridge connected to CHC₆H₅:

CH₂ and CH each bonded to O, O, bridged to CHC₆H₅

Since the aliphatic diazo derivatives readily lost nitrogen with the formation of an unsaturated derivative it was necessary to test whether on reduction with aluminum amalgam the desoxy derivative was formed through the intermediate formation of the unsaturated derivative.   In view of this possibility it was attempted to reduce with aluminum amalgam the 5,6-benzylidene-1-amino-2,3-anhydrogluconate.   The result, however, was negative.

EXPERIMENTAL.

Reduction of Benzylidene-1-Ethyl-2-Diazogluconate (Mannonate).—The reduction was accomplished by means of freshly prepared aluminum amalgam in a solution of the diazo derivative in isopropyl alcohol.   The diazo derivative (10 gm.) is dissolved on the boiling water bath in the solvent (100 cc.) and to the warm solution 1.0 gm. of freshly prepared amalgam is added.   A mild evolution of gas sets in immediately and subsides in about 3 hours.   The reaction product is then filtered and a new lot of amalgam

[2] Staudinger, H., Gaule, A., and Siegwart, J., *Helvetica Chim. Acta*, 1921, iv, 212.

* The substance should be graphically presented in this form and not as on page 152, Vol. liii of this *Journal*.   The error was overlooked in proof-reading.

added. As a rule three renewals of the amalgam were required to complete the reduction. Evolution of ammonia gas was noted soon after the beginning of the reaction. After the reduction was completed the clear filtrate from the aluminum was concentrated under diminished pressure, and the residue, at first syrupy, on standing turned into a crystalline mass. This was transferred into an evaporating dish by means of warm ether in which the residue dissolved readily. On standing it soon crystallized in long prismatic needles. The yield of crystalline material was 1.0 gm. for each 10 gm. of the original material. From the mother liquor further crops could be obtained. This, however, was not done, so as not to affect the maximum yield of the chitosaminic acid derivative contained in the filtrate.

The crystals were found insoluble in water and in dilute aqueous mineral acids, but very soluble in ether, alcohol, methyl alcohol, and acetone. For analysis it was recrystallized out of ether. It melted at 124°C. (corrected) and analyzed as follows:

0.1014 gm. substance: 0.2272 gm. CO_2 and 0.0620 gm. H_2O.
$C_{15}H_{20}O_6$. Calculated.   C 60.81, H 6.76.
Found.   " 61.10, " 6.84.

The rotation of the substance was:

$$[\alpha]_D^{20} = \frac{-0.56° \times 100}{1 \times 2} = -28.0°$$

Thus this substance was identical with the benzylidene-1-ethyl-2-desoxygluconate described in a previous publication. The mother liquor contained the derivative of chitosaminic acid. The original mother liquor was taken up in 100 cc. of 2 per cent sulfuric acid and placed on a water bath until all the ether was evaporated and all the benzaldehyde settled out on the bottom of the flask as a yellow oil. The reaction product was cooled, the benzaldehyde extracted with ether, and the remaining aqueous layer allowed to stand over night with a slight excess of baryta water. The excess of barium was then removed quantitatively with sulfuric acid. The combined mother liquors of two experiments of 10.0 gm. each, were analyzed for amino nitrogen and showed after 4 minutes shaking 0.090 gm. of nitrogen, equivalent to

1.26 gm. of chitosaminic acid. This aqueous solution was con-
centrated under diminished pressure to a thick syrup, which was
transferred to a small beaker by means of a little methyl alcohol.
To the solution methyl alcohol was added until a small sticky
precipitate appeared. The beaker was placed on a water bath
and the contents were stirred until the chitosaminic acid began to
crystallize. The yield of the crystalline material was 0.60 gm.
This material without further purification had the optical rotation:
$[\alpha]_D^{20} = -15.0°$ thus showing that it consisted only of chitosaminic
acid without any admixture of its epimer. For purification the
combined material obtained from 60 gm. diazo compound was
recrystallized twice from dilute methyl alcohol. The final sub-
stance analyzed as follows:

0.0200 gm. were dissolved in 5 cc. of water.
2 cc. of this solution: (Van Slyke) 1.07 cc. N_2 at 26°C., 757.4 mm.
$$C_6H_{13}NO_6.\quad \text{Calculated.}\quad N\ 7.18.$$
$$\text{Found.}\qquad "\ 7.26.$$

Its optical rotation was:

$$[\alpha]_D^{20} = \frac{-0.15° \times 100}{1 \times 1} = -15.0°$$

In order to test the purity of the diazo derivative the substance
(20.0 gm.) was hydrolyzed with dilute sulfuric acid and treated
in exactly the same manner as described above. The resulting
solution showed the presence of 0.003 gm. of nitrogen, practically
a negligible quantity.

*Reduction of Benzylidene-1-Amino-2, 3-Anhydrogluconate (Man-
nonate).*—The substance (5.0 gm.) was dissolved in 300 cc. of
isopropyl alcohol and freshly prepared aluminum amalgam was
added (1.0 gm.). Only a slight evolution of gas was noted.
After 2 hours the reaction product was filtered and to the filtrate
another portion of aluminum amalgam was added. The flask
was allowed to stand over night, and the clear filtrate concen-
trated. The residue crystallized in the distilling flask. The
residue was dissolved in hot ether and transferred to a crystalliz-
ing dish. The substance crystallized very soon and was filtered
off after several hours. The mother liquor on evaporation gave

a very insignificant residue. The crystalline material melted at 230°C. and analyzed as follows:

0.1068 gm. substance: 0.2308 gm. CO_2 and 0.0564 gm. H_2O.
$C_{13}H_{15}NO_5$. Calculated. C 58.84, H 5.70.
Found. " 58.90, " 5.90.

The optical rotation was:

$$[\alpha]_D^{20} = \frac{-1.32° \times 100}{1 \times 2} = -66.0°$$

INDEX TO AUTHORS.

F

Falk, K. George, and **McGuire, Grace.** Studies on enzyme action. XXI. Banana gel and banana sucrase, 655

Folin, Otto. A system of blood analysis. Supplement IV. A revision of the method for determining uric acid, 153

Fred, E. B. See PETERSON, FRED, and SCHMIDT, 19

G

Gaebler, Oliver Henry. See HARDING and GAEBLER, 579

Greenwald, Isidor, and **Lewman, Gertrude.** The determination of the titratable alkali of the blood, 263
—. The supposed relation between alkalosis and tetany, 285
—, and **Gross, Joseph.** Some sources of error in the determination of chlorides in blood and similar material, 589

Gross, Erwin G., and **Underhill, Frank P.** The metabolism of inorganic salts. I. The organic ion balance of the blood in parathyroid tetany, 105

Gross, Joseph. See GREENWALD and GROSS, 589

H

Hammett, F. S., and **Adams, E. T.** Note on a colorimetric method for the determination of small amounts of magnesium, 565

Harding, Victor John, and **Gaebler, Oliver Henry.** On the constancy of the creatine-creatinine excretion in children on a high protein diet, 579

Harrop, G. A. See BENEDICT and HARROP, 443

Hart, E. B., Steenbock, H., Hoppert, C. A., Bethke, R. M., and **Humphrey, G. C.** Dietary factors influencing calcium assimilation. III. The comparative efficiency of timothy hay, alfalfa hay, and timothy hay plus calcium phosphate (steamed bone meal) in maintaining calcium and phosphorus equilibrium in milking cows, 75

Hastings, A. B. See AUSTIN, CULLEN, HASTINGS, McLEAN, PETERS, and VAN SLYKE, 121
—. See VAN SLYKE, HASTINGS, HEIDELBERGER, and NEILL, 481
—. See VAN SLYKE, HASTINGS, and NEILL, 507

Hedin, S. G. On the proteolytic enzymes of the spleen, 177

Heidelberger, Michael. See JACOBS and HEIDELBERGER, 253
—. See VAN SLYKE, HASTINGS, HEIDELBERGER, and NEILL, 481

Hopkins, F. Gowland, and **Dixon, M.** On glutathione. II. A thermostable oxidation-reduction system, 527

Hoppert, C. A. See HART, STEENBOCK, HOPPERT, BETHKE, and HUMPHREY, 75

JOURNAL OF BACTERIOLOGY

OFFICIAL ORGAN OF THE SOCIETY OF AMERICAN BACTERIOLOGISTS

DEVOTED TO THE ADVANCEMENT AND DISSEMINATION OF KNOWLEDGE IN REGARD TO THE BACTERIA AND OTHER MICRO-ORGANISMS

CONTENTS

Subscription Order for the Journal of Bacteriology

WILLIAMS & WILKINS COMPANY, MOUNT ROYAL AND GUILFORD AVENUES, BALTIMORE, MD., U. S. A.

Check or Order for the JOURNAL OF BACTERIOLOGY { Vol. I, Vol. II, Vol. III, Vol. IV, Vol. V. } Check Volumes Desired is enclosed to cover subscription.

Name ....

(Please state whether Dr., Prof., etc.)

Address ....

JOURNAL OF IMMUNOLOGY

OFFICIAL PUBLICATION OF

THE SOCIETY FOR SEROLOGY AND HEMATOLOGQ AND
THE AMERICAN ASSOCIATION OF IMMUNOLOGISTS

Edited by ARTHUR F. COCA

Subscription Order for the Journal of Immunology

WILLIAMS & WILKINS COMPANY, MOUNT ROYAL AND GUILFORD AVENUES, BALTIMORE, MD., U. S. A.

$\left.\begin{matrix}\text{I}\\\text{We}\end{matrix}\right\}$ enclose $\left.\right\}$ $_____ $\left.\right\}$ for subscription to the current volume of JOURNAL OF IMMUNOLOGY.

Please send Back Volumes _____

Name..
(Please state whether Dr., Prof., etc.)

Address..

MEDICINE

A NEW PERIODICAL

Publishing Analytical Reviews of General Medicine, Neurology, Pediatrics

500 PAGES TO THE VOLUME

PUBLISHED QUARTERLY

MEDICINE will interest:

TEACHERS, who wish to keep in touch with the methods and results of medical work in their own and other fields.

CLINICIANS, who wish the advantage of having immediately available, in convenient and readable form, critical digests of the recent views on general medicine, neurology and pediatrics.

LABORATORY WORKERS, who require a useful reference work that will give systematic and careful presentations of subjects of important medical interest.

SUBSCRIPTION PRICE, NET POSTPAID

$5.00, United States, Mexico, Cuba $5.25, Canada $5.50, other countries

The Journal of General Physiology

Edited by

JACQUES LOEB, New York, N. Y.

W. J. V. OSTERHOUT, Cambridge, Mass.

CONTENTS OF VOL. V, No. 2, NOVEMBER 20, 1922

Published by

The Rockefeller Institute for Medical Research
Avenue A and 66th Street, New York, N. Y.

THE AMERICAN JOURNAL

OF

TROPICAL MEDICINE

OFFICIAL ORGAN OF THE
AMERICAN SOCIETY OF TROPICAL MEDICINE

Issued Bimonthly; six issues a year, 85 pages, approximately, an issue; one volume a year is planned at present.

Price, net postpaid: $5.00, United States and dependencies, Mexico, Cuba; $5.25, Canada; $5.50, other countries.

Order from

WILLIAMS & WILKINS COMPANY
Publishers of Scientific Journals and Books
BALTIMORE, U. S. A.

'te

On

ce

ary
ib-
con-

ood
ein
sue,
ion,
log-

tise.

keep

OLOR

born
nds;
nent.